OLYMPUS

User Manual

cellSens[Ver. 3.2]

IMAGING SOFTWARE

For research and education

This software has been designed for research and educational purposes.

cellSens 3.2_Zambesi_00_en

Contents

1. About the documentation for your software

The documentation for your software consists of several parts: the installation manual, the online help, and PDF manuals which were installed together with your software.

Where do you find which information?

The installation manual is delivered with your software. There, you can find the system requirements. Additionally, you can find out how to install and configure your software.

In the manual, you will find both an introduction to the product and an explanation of the user interface. By using the extensive step-by-step instructions you can quickly learn the most important procedures for using this software.

In the online help, you can find detailed help for all elements of your software. An individual help topic is available for every command, every toolbar, every tool window and every dialog box.

New users are advised to use the manual to introduce themselves to the product and to use the online help for more detailed questions at a later date.

Writing convention used in the documentation

In this documentation, the term "your software" will be used for cellSens.

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Example images

The DVD that comes with your software contains, among a lot of other data, also images that show different examples of use for your software. You can load these so-called example images from the DVD. However, in many cases, installing the example images on your local hard disk or on a network drive is more helpful. Then the example images will always be available, no matter where the DVD with the software currently is.

Note: Your software's user documentation often refers to these example images. You can directly follow some step-by-step instructions when you load the corresponding example image.

You can open and view the example images with your software. Additionally, you can use the example images to test some of your software's functions, for example, the automatic image analysis, the image processing or the report creation.

Due to the fact that the example images also contain multi-dimensional images like Z-stacks or time stacks, making use of them enables you to quickly load images that require more complex acquisition settings.

Installing example images

You can install the example images after you've installed the software, or at any later point in time.

To do so, insert the DVD that contains the software into the DVD drive. If the installation wizard starts, browse to the directory that contains the example images and install them.

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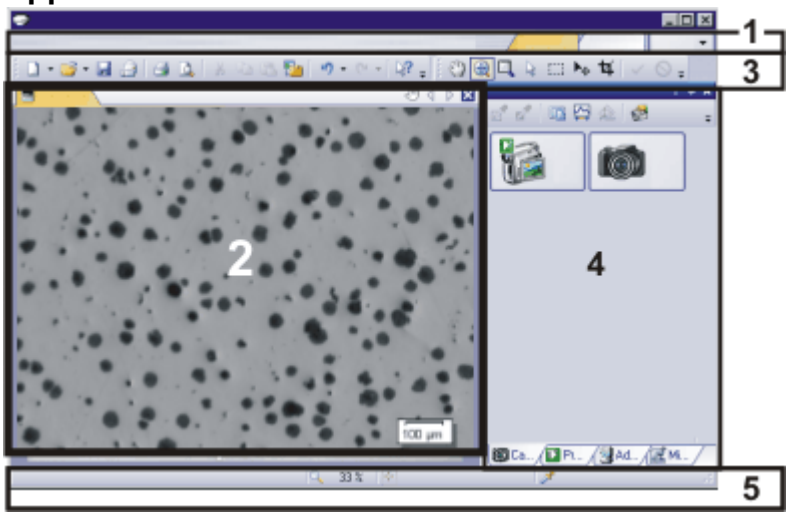
2. Overview - User interface

The graphical user interface determines your software's appearance. It specifies which menus there are, how the individual functions can be called up, how and where data, for example images, is displayed, and much more. In the following, the basic elements of the user interface are described.

Note: Your software's user interface can be adapted to suit the requirements of individual users and tasks. You can, e.g., configure the toolbars, create new layouts, or modify the document group in such a way that several images can be displayed at the same time.

Appearance of the user interface



The illustration shows the schematic user interface with its basic elements.

(**1**) Menu bar
(**2**) Document group
(**3**) Toolbars
(**4**) Tool windows
(**5**) Status bar

(1) Menu bar

You can call up many commands by using the corresponding menu. Your software's menu bar can be configured to suit your requirements. Use the *Tools > Customization > Start Customize Mode* command to add menus, modify, or delete them.

(2) Document group

The document group contains all loaded documents. These can be of all supported document types.
When you start your software, the document group is empty. While you use your software it gets filled - e.g., when you load or acquire images, or perform various image processing operations to change the source image and create a new one.

(3) Toolbars

Commands you use frequently are linked to a button providing you with quick and easy access to these functions. Please note, that there are many functions which are only accessible via a toolbar, for example, the drawing functions required for annotating an image. Use the *Tools > Customization > Start Customize Mode* command to modify a toolbar's appearance to suit your requirements.

(4) Tool windows

Tool windows combine functions into groups. These may be very different functions. For example, in the *Properties* tool window, you can find all the information available on the active document.

In contrast to dialog boxes, tool windows remain visible on the user interface as long as they are switched on. That gives you access to the settings in the tool windows at all times.

(5) Status bar

The status bar contains a large amount of information, for example a brief description of each function. Simply move the mouse pointer over the command or button for this information.

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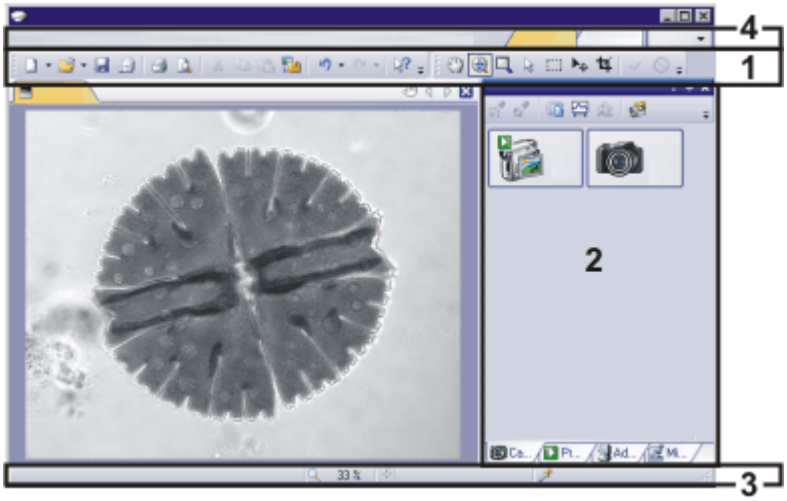
2.1. Overview - Layouts

What is a layout?

Your software's user interface is to a great extent configurable, so that it can easily be adapted to meet the requirements of individual users or of different tasks. You can define a so-called layout that is suitable for the task on hand. A layout is an arrangement of the control elements on your monitor that is optimal for the task on hand. In any layout, only the software functions that are important in respect to this layout will be available.

Example: The *Camera Control* tool window is only of importance when you acquire images. When instead of that, you want to measure images, you don't need that tool window.
That's why the *Acquisition* layout contains the *Camera Control* tool window, whereas in the *Processing* layout it's hidden.

Which elements of the user interface belong to the layout?

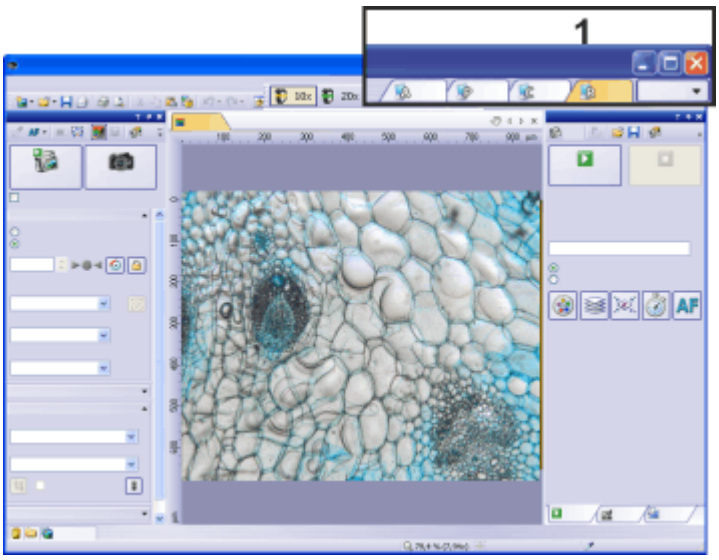


The illustration shows you the elements of the user interface that belong to the layout. The layout saves the element's size and position, regardless of whether they have been shown or hidden. When, for example, you have brought the *Windows* toolbar into a layout, it will only be available for this one layout.

(**1**) Toolbars
(**2**) Tool windows
(**3**) Status bar
(**4**) Menu bar

Switching to a layout

To switch backwards and forwards between different layouts, click on the right-hand side in the menu bar on the name of the layout you want, or use the *View > Layout* command.



You can find a tab for each layout at the top right of the menu bar (**1**). Click one of the tabs to switch to the corresponding layout.

Which predefined layouts are there?

For important tasks several layouts have already been defined. The following layouts are available:

Layout	Application
Acquisition	Acquiring images
Well Navigation	Acquiring images on a well plate
Processing	Processing images
Count and Measure	Measuring images
Report	Generating reports
Deep Learning	Training neural networks
Simple	Easily acquiring images and discussing them

Restoring layouts

In contrast to your own layouts, predefined layouts can't be deleted. Therefore, you can always restore a predefined layout back to its originally defined form. To do this, select the predefined layout, and use the *View > Layout > Reset Current Layout* command.

Saving function sets in a layout

In the *My Functions* tool window, you assign software functions that you use frequently to a function set, and arrange the functions in their own tool window. You can arrange the tool windows on the user interface for your convenience and save them in a layout so that you can access them at any time.

Using the Simple layout

The *Simple* layout is a special layout that supports you in your day-to-day work. The layout facilitates acquiring snapshots, basic image processing, and sharing.

When you are working with the *Simple* layout, all of the other layouts can no longer accessed.

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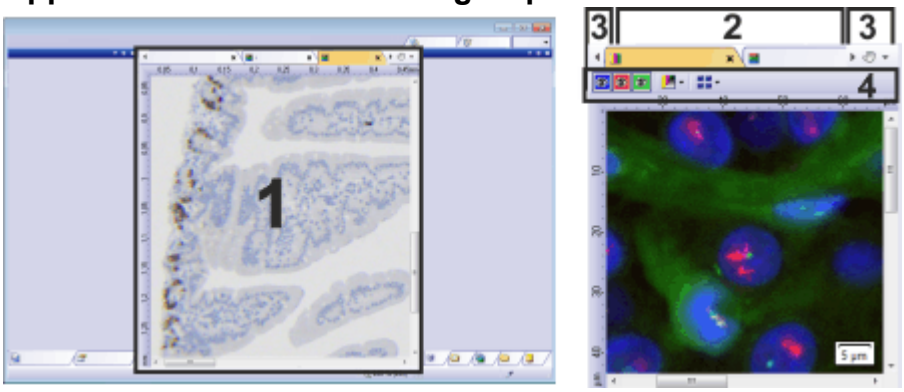
2.2. Document group

The document group contains all loaded documents. As a rule, images will be loaded. You can also find other types of documents in the document group, charts for example.

Appearance of the document group



(**1**) Document group in the user interface
(**2**) Document bar in the document group
(**3**) Buttons in the document bar
(**4**) Toolbar in the image window

2.2.1. Document group in the user interface

You will find the document group in the middle of the user interface. In it you will find all of the documents that have been loaded, and of course also all of the images that have been acquired. The live-image and the images resulting from any image processing function are also displayed there.

Note: At the same time, up to 150 documents can be loaded in the document group.

2.2.2. Document bar in the document group

The document bar is the document group's header.

For every loaded document, an individual tab showing the document name will be set up in the document group. Click the name of a document in the document bar to have this document displayed in the document group. The name of the active document will be shown in color. Each type of document is identified by its own icon.

At the top right of each tab, a small [**x**] button is located. Click the button with the cross to close the document. If it has not yet been saved, the *Unsaved Documents* dialog box will open. You can then decide whether or not you still need the data.

2.2.3. Buttons in the document bar

The document bar contains several buttons, on the left and on the right.

Hand button

Click the button with a hand on it to extract the document group from the user interface. In this way you will create a document window that you can freely position or change in size.

If you would like to merge two document groups, click the button with the hand in one of the two document groups. With the left mouse button depressed, drag the document group with all the files loaded in it, onto an existing one.

Prerequisite: You can only position document groups as you wish when you are in the expert mode. In standard mode the button with the hand is not available.

Arrow buttons

You can find two arrow buttons at the top left and the top right of the document group.

When your software starts, the arrow buttons are inactive. The arrow buttons will only become active when you have loaded so many documents that all of their names can no longer be displayed in the document group.

If you have loaded so many images that all of their names can no longer be displayed in the document group, click one of the two arrows. This scrolls the fields with the document names to the left or to the right. That will enable you to see the documents that were previously not shown.

List of loaded documents

Click the small arrow on the right to open a list of all of the loaded documents. If you are using more than one document group, the loaded documents are sorted by document group. A horizontal line divides the document groups from each other.

Left click the document that you want to have displayed on your monitor.

Alternatively, you can use the *Documents* tool window or the *Gallery* tool window to get an overview of the documents that have been loaded.

2.2.4. Toolbar in the image window

With some image types, the image window contains a toolbar. There are some other document types with their own navigation bar directly in the image window. One example is a report instruction or an experiment plan.

Image type	Buttons in the image window		
All images		Adjust Display	Click this button to change the image contrast.
Multi-layer images		*Set layer visibility*	Use this button to make the layers appear or disappear.
Multi-channel images		Navigation bar	Multi-dimensional images, time stacks for example, have their own navigation bar directly in the image window. Use this navigation bar to set or to change how a multi-dimensional image is to be displayed on your monitor.
Z-stack Time stacks		Navigation bar	
Multi-channel images Z-stacks Time stacks		Image window views	There can be more than one view for the same image. For example, with an image series you can display in the image window either an individual image or an overview of all of the individual images. There is a menu with all of the image window view options for the active image on the image window's toolbar.

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2.3. Tool Windows

What is a tool window?

Tool windows combine functions into groups. These may be very different functions. For example, in the *Properties* tool window, you can find all the information available on the active document.

In contrast to dialog boxes, tool windows keep visible on the user interface as long as they are switched on. That gives you access to the settings in the tool windows at all times.

Showing and hiding tool windows

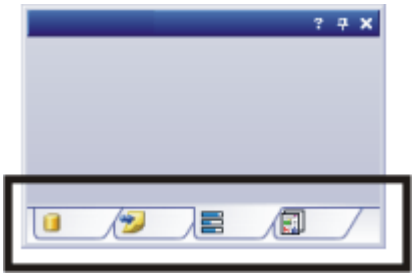
Which tool windows are shown by default depends on the layout you have chosen. You can, at any time, make specific tool windows appear and disappear manually. To do so, use the *View > Tool Windows* command.

2.3.1. Position of the tool windows

The user interface is to a large degree configurable. For this reason, tool windows can be docked, freely positioned, or integrated in document groups.

Docked tool windows

Tool windows can be docked to the left or right of the document window, or below it. To save space, several tool windows may lie on top of each other. They are then arranged as tabs. In this case, activate the required tool window by clicking the title of the corresponding tab below the window.



Freely positioned tool windows

Prerequisite: You can only position tool windows as you wish when you are in the expert mode.

You can at any time float a tool window. To release a tool window from its docked position, click on its header with your left mouse button. Then, while pressing the left mouse button, drag the tool window to wherever you want it.

Saving the tool window's position

Tool windows and their positions are saved together with the layout and are available at the same position the next time you start your software. A return to the original layout using the *View > Layout > Reset Current Layout* command will have the result that only the tool windows that are defined by default for this layout will be displayed.

2.3.2. Buttons in the header

In the header of every tool window, you will find the three buttons *Help*, *Enable Auto Hide*, and *Close*.

Click the *Help* button to open the online help for the tool window.

Click the *Enable Auto Hide* button to minimize the tool window.

Click the *Close* button to hide the tool window. You can show it again at any time using the *View > Tool Windows* command.

2.3.3. Context menu of the header

To open a context menu, right click a tool window's header. The context menu can contain the *Auto·Hide*, *Document Mode*, and *Transparency* commands.

Additionally, the context menu contains a list of all of the tool windows that are available. Every tool window is identified by its own icon. The icons of the currently displayed tool windows will appear clicked. You can recognize this status by the icon's background color. Use this list to make tool windows appear.

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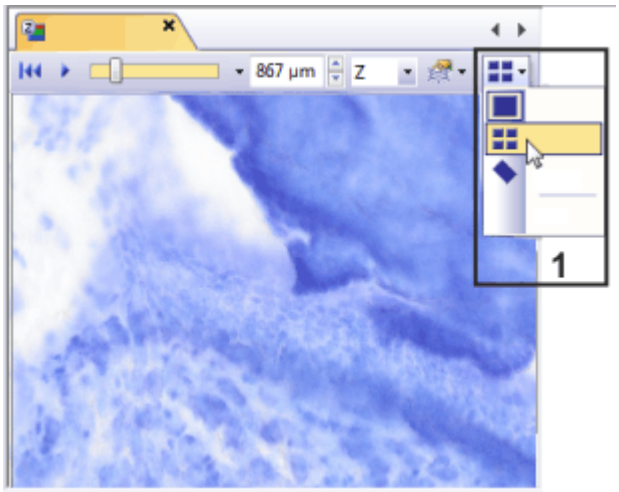
2.4. Image window views

All of the images that are loaded in your software are displayed in the image window. When working with some image types, all multi-dimensional images for example, you can choose between different views of the image in the image window. In this case a navigation bar is displayed in the image window. Click the small arrow next to the last button on this navigation bar to open a menu with commands you can use with image window views. In it, you can select the image window view you want and also edit the settings for some views.



The illustration shows the context menu with all of the available image window views (**1**).

The button's appearance

The button is configured to enable you to switch between single frame view and a different image window view with a click of the mouse.
Click the button to switch to the image window view that is currently shown as an icon on the button. Every image window view has its own icon.

2.4.1. Overview - Image window views

Single Frame View

By default you will find yourself in single frame view. In the single frame view, only one image will be shown in the image window.

Tile View

Use the tile view to attain an overview of all of the individual images that make up a multi-dimensional image. In this view, you can also select individual images.

Slice View

Use the *Slice View* image window view to look at any cross sections of an image series you want. The *Slice View* tool window offers various possibilities for configuring this view.

Voxel View

You can display a Z-stack as a 3D object. To do this, use the *Voxel View* image window view and the *Voxel View* tool window.

Projection Views

For image series, e.g. Z-stacks and time stacks, a single projection image can be calculated from all of the frames that is representative for the whole multi-dimensional image. The available projection images differ in the calculation algorithm. For example, if you use the maximum intensity projection you will, from all frames, only see the pixels with the highest intensity values.

EFI Projection

For Z-stacks an EFI projection is available. The EFI projection uses a series of differently focused separate images (Focus series) to calculate a resulting image (EFI image), that is focused in all of its parts. You can display a Z-stack as a 3D object. To do this, use the *Voxel View* image window view and the *Voxel View* tool window.

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2.5. Working with documents

You can choose from a number of possibilities when you want to open, save, or close documents. As a rule, these documents will be images. In addition, your software supports other document types as well.

Saving documents

You should always save important documents immediately following their acquisition. You can recognize documents that have not been saved by the star icon after the document's name.

There are a number of ways in which you can save documents.

1. To save a single document, activate the document in the document group. Then use the *File > Save As* command or press [Ctrl + S] on your keyboard.
2. Use the *Documents* tool window.
   Select the desired document and use the *Save* command in the context menu. For the selection of documents, the standard MS-Windows conventions for multiple selection are valid.
3. Use the *Gallery* tool window.
   Select the desired document and use the *Save* command in the context menu. For the selection of documents, the standard MS-Windows conventions for multiple selection are valid.
4. Save your documents in a database. That enables you to store all manner of data that belongs together in one location. Search and filter functions make it quick and easy to locate saved documents.

Automatic save

1. When you exit your software, all data that has not yet been saved will be listed in the *Unsaved Documents* dialog box. This gives you the chance to decide which document you still want to save.
   - When you exit your software, all data that has not yet been saved will be listed in the *Unsaved Documents* dialog box. This gives you the chance to decide which document you still want to save.
   - With some acquisition processes, the acquired images will be automatically saved after the acquisition has finished.
2. You can also configure your software in such a way that all images are saved automatically after image acquisition. To do so, use the *Acquisition Settings > Saving* dialog box.

   Here, you can also configure your software in such a way that all images are automatically saved in a database after the image acquisition.

Closing documents

There are a number of ways in which you can close documents.

1. Use the *Documents* tool window.
   Select the document you want and use the *Close* command in the context menu.

For the selection of documents, the standard MS-Windows conventions for multiple selection are valid.

2. To close a single document, activate the document in the document group and use the *File > Close* command. Alternatively, you can click the button with the cross [**x**]. You can find this button at the top right of the document tab next to the document name.
3. Use the *Gallery* tool window.
   Select the document you want and use the *Close* command in the context menu.
   For the selection of documents, the standard MS-Windows conventions for multiple selection are valid.

Closing all documents

To close all loaded documents use the *Close All* command or press [Ctrl + Alt + W] on your keyboard. You will find this command in the *File* menu, and in both the *Documents* and the *Gallery* tool windows' context menu.

Closing a document immediately

To close a document immediately without a query, close it with the [Shift] key depressed. Data you have not saved will be lost.

Opening documents

There are a number of ways in which you can open or load documents.

1. Use the *File > Open* command.
2. Drag the document you want, directly out of the MS-Windows Explorer, onto your software's document group.
3. To load documents from a database into the document group, use the *Database > Load Documents* command.

Note: At the same time, up to 150 documents can be loaded in the document group.

Generating a test image

If you want to get used to your software, then sometimes any image suffices to try out a function.

Press [Ctrl + Shift + Alt + T] to generate a color test image.
With the [Ctrl + Alt + T] shortcut, you can generate a test image that is made up of 256 gray values.

Activating documents in the document group

There are several ways to activate one of the documents that has been loaded into the document group and thus display it on your monitor.

1. Use the *Documents* tool window. Click the desired document there.
2. Use the *Gallery* tool window. Click the desired document there.
3. Click the title of the desired document in the document group.
4. To open a list with all currently loaded documents, use the [Ctrl + Tab] shortcut. Left click the document that you want to have displayed on your monitor.
5. Click the small arrow at the top right of the document group to open a list of all of the loaded documents. Left click the document that you want to have displayed on your monitor.
6. Use the keyboard shortcut [Ctrl + F6] or [Ctrl + Shift + F6], to have the next document in the document group displayed. With this keyboard shortcut you can display all of the loaded documents one after the other.
7. In the *Window* menu, you can find a list of all of the documents that have been loaded. Select the document you want from this list.

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3. Acquiring snapshots and discussing them

If you want to acquire snapshots with your software and to discuss them with other people, your software provides a special layout for doing this. The *Simple* layout facilitates acquiring snapshots, basic image processing, and sharing.

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3.1. Layout - Simple

Activating a layout

In the *Simple Layout* dialog box, you can activate the *Simple* layout the first time you open your software. To do so, click the *Select Simple Layout* button.

If the *Simple Layout* dialog box doesn't appear the first time you open your software, use the *Tools > Options* command. Select the *Simple Layout > General* entry in the tree view. Select the *Display the dialog for selecting the layout at startup* check box. Restart your software.

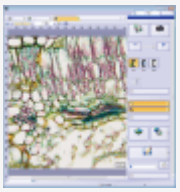
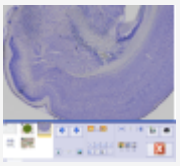
The Simple layout and other layouts

When you are using the *Simple* layout, all of the other layouts can no longer accessed. If you want to use a different layout, deactivate the *Simple* layout.

Use the *Tools > Options* command. Select the *Simple Layout > General* entry in the tree view. Click the *Deactivate* button.

The best user interfaces for different tasks

The *Simple* layout facilitates acquiring snapshots, basic image processing, and sharing. All of the software functions that are not needed for these functions are not shown, to keep the layout simple.

(1)		Acquiring snapshots	When you open the *Simple* layout, your software's user interface is arranged to facilitate acquiring snapshots easily.
(2)		Processing images	You can find different groups of functions in the *Smooth Control* tool window. In the *Image* group, click the *Processing* button to process an image that has been acquired.
(3)		Labeling and discussing images	Use conference mode to view images, to label them, and to discuss them with colleagues. To open the *Conference Mode*, click the *Conference* button in the *Smooth Control* tool window.

Changing the Simple layout

You can change the *Simple* layout just like any other layout. You can use the commands in the *View > Tool Windows* menu to show other tool windows on the user interface. Changes made in layouts will be automatically saved. When you close your software, then restart it, you won't see the *Simple* layout as it was defined, but instead, the layout as you have changed it.

Use the *View > Layout > Reset Current Layout* command to restore the layout to its default configuration.

The tool windows lie one over the other. Activate the required tool window by clicking the title of the corresponding tab below the window.

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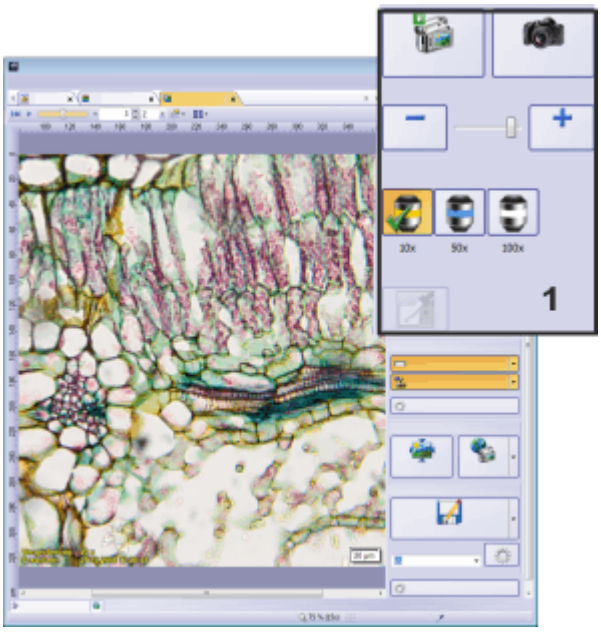
3.2. Working with the Simple layout

If you want to acquire snapshots with your software and to discuss them with other people, your software provides a special layout for doing this. The *Simple* layout facilitates acquiring snapshots, basic image processing, and sharing.

3.2.1. Acquiring snapshots

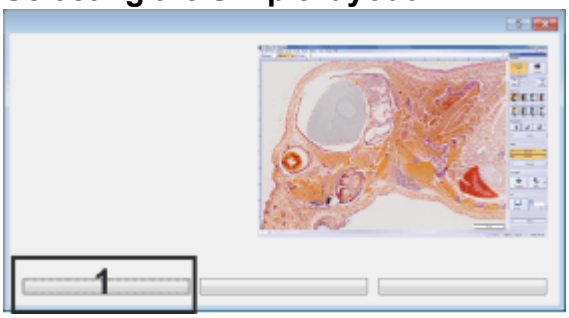
Task: You want to acquire several images of a sample, one after the other.

Prerequisite: The system has already been configured and calibrated.



In the *Simple* layout, you can find the *Smooth Control* tool window to the right of the document group. It contains the *Acquisition* group (**1**).

Selecting the Simple layout



When you start your software for the first time, the *Simple Layout* dialog box appears. Click the *Select Simple Layout* (**1**) button if you want to work with the *Simple* layout.

1. When your software opens, click the *Select Simple Layout* button.
2. If the *Simple Layout* dialog box doesn't open when you start your software:

   Use the *Tools > Options* command.

   Select the *Simple Layout > General* entry in the tree view.

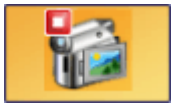
   Select the *Display the dialog for selecting the layout at startup* check box. Restart your software.

   - The *Simple* layout arranges your software's user interface to facilitate acquiring snapshots easily.

Switching on the live-image

3. Click the *Live* button.

- A new window called *Live (active)* automatically opens for the live-image.
- The live-image will be shown in the live window.
- The *Live* button changes its appearance in live-mode. This enables you to immediately recognize that you are in live-mode.



- The *White balance* button is now activated.

4. With the microscope, go to the required position on the sample.

Selecting an objective

5. Click the button with the objective that you use for the image acquisition.

- There is an info stamp with the magnification at the bottom left of the image window.

  If the info stamp is not shown, click the *Info Stamp* button.
- You can see the scale bar at the bottom right of the image window. It corresponds to the selected magnification.

  If the scale bar is not shown, click the *Scale Bar* button.

Setting the image quality

6. Bring the sample into focus.
7. Make sure that you are using automatic exposure. Click the *Settings* button at the bottom of the *Acquisition* group. Click the *Default* button and then the *Close* button.

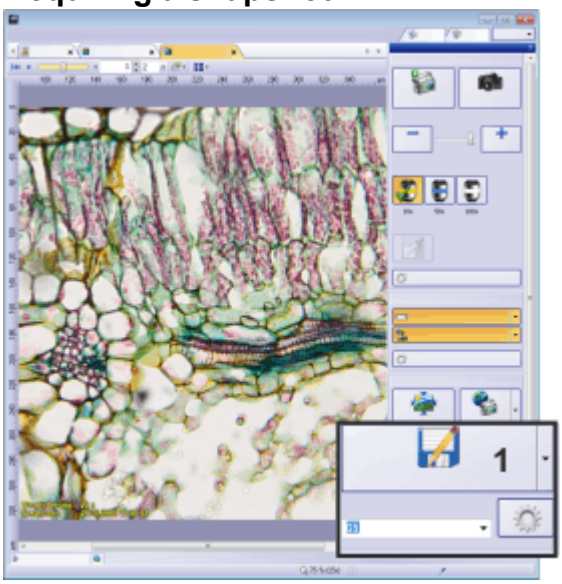
8. Check the exposure time. Use the [-] and[+] buttons to make the image darker or brighter.

9. Check the color reproduction.

   If necessary, carry out a white balance. To do this, click the *White balance* button.

10. Move the pointer onto the top left-hand corner of an image segment that is to be displayed in white. With the left mouse button depressed draw a rectangle.
    - The white balance will be performed immediately. This means that the individual image colors will be adapted in such a way that the image segment you've selected will be displayed in white. You can see the results immediately in the image.

Acquiring a snapshot



You can find the *Files* group at the bottom of the *Smooth Control* tool window (**1**).

11. Specify a name for the image. You enter the first part of the name in the *Text* field. This part is automatically assigned when the image is acquired. You can find the *Text* field in the *Files* group in the *Smooth Control* tool window.

    Replace the default text *Image*, for example with *Sample-AB*.

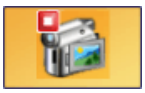
12. Click the *Snapshot* button.

- A new image window for the image that has been acquired appears in the document group. The first acquired image is called *Sample-AB_01*.
- The software automatically goes back to live-mode.
- The live-image is displayed in the document group.

13. Acquire additional images. Go to a different position on the sample and refocus. Change the magnification or the exposure time. You can control all of these functions in live-mode.

Leaving live-mode and saving images

14. Click the *Live* button again to leave live-mode.



- The live window closes.
- The last image to be acquired appears in the image window.

15. Click the *Save As* button to save the active image as a file. Select the drive and directory in which you want to save this document. Enter a file name. Use the recommended TIF or VSI file format.

- Please note: When the live window is active, the *Save As* button opens the *Acquisition Settings > Saving* dialog box. In this dialog box, you can specify whether and how to automatically save images after they have been acquired.

16. Close the image window with the saved image. There is a small button [**x**] in the image window's tab. Click the button with the cross to close the image window.
    - The next image now appears in the image window.

17. Decide whether you want to save each image or not.
    - If you don't want to save each image individually, use the *File > Close All* command. Now, all of the images that have not yet been saved will be listed in the *Unsaved Documents* dialog box. This gives you the chance to decide which document you still want to save.
    - You can also save all of the snapshots automatically after they are acquired.

      To do this, in live-mode, click the *Save As* button to open the *Acquisition Settings > Saving* dialog box.

      Select the *File system* entry from the *Automatic save > Destination* list. Specify the destination location for saving your documents.

      Now, every image that is acquired will be automatically saved to the specified destination location.

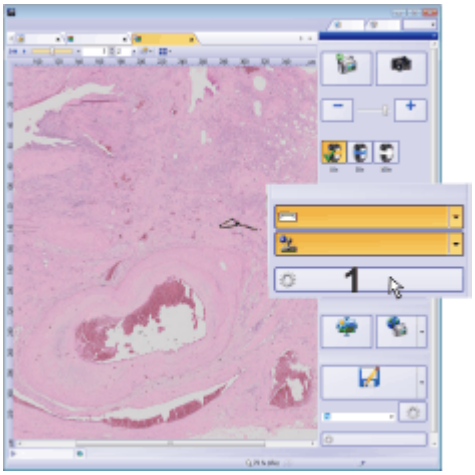
3.2.2. Processing images

Task: Increase the contrast in an image. Also crop the image so that only the segment of the image that you are interested in is displayed.

Changing the user interface

1. The *Image* group in the *Smooth Control* tool window gives you access to several image processing functions. In the *Image* group, click the *Processing* button.

    - The *Smooth Control* tool window changes its appearance. In the tool window, you can now find several functions for changing the image you acquired. The image acquisition and save commands are hidden.
    - At the bottom left-hand edge of the user interface, you can see two tabs with the icons for the *Measurement and ROI* tool window and the *Gallery* tool window.

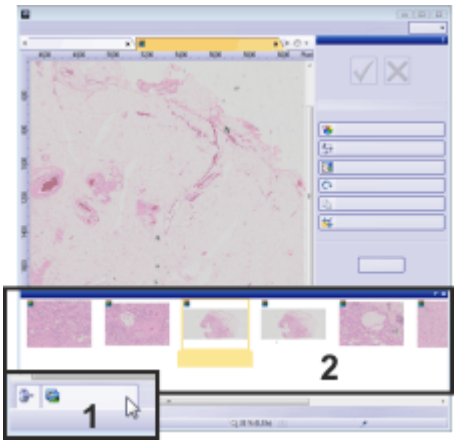


Click the *Processing* button (**1**) to change the appearance of the *Smooth Control* tool window.

Selecting an image

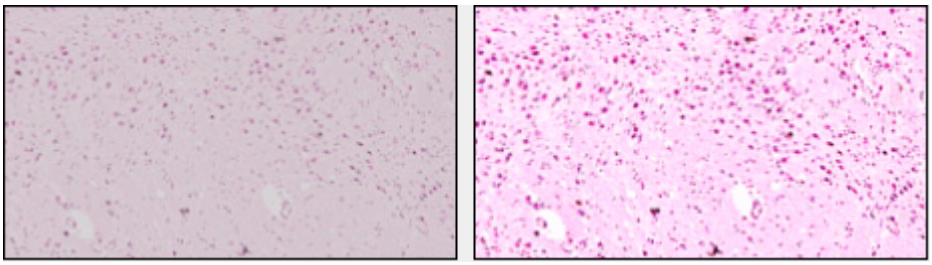
2. Hover the mouse over the icon for the *Gallery* tab. You can find the icon at the bottom left-hand edge of the user interface.
    - A gallery opens with all of the images that are loaded.

3. In the gallery, click the image you want to process.
   - The selected image is now displayed in the image window. All of the image processing functions take effect on the active image.



Open the gallery (**1**) and select the image that you want to process (**2**).

4. In the *Smooth Control* tool window, click the *Adjust Intensity...* button.
   - The image processing dialog box opens.
   - The same image segment is displayed twice in the dialog box. The first one shown is the source image. The second is the image that results when the current parameters are applied.
5. Clear the *Create new document as output* check box. Now, the *Adjust Intensity* command will change the source image. No new image document will be created.
6. Change the image processing operation's parameters. You can decrease the gamma value and increase the brightness, for example.
   - After every change that is made in a parameter, the operation will be immediately applied to the source image, and the resulting image will be shown in the preview window.
7. When you have found the optimal parameters, click the *OK* button to change the contrast in the image as shown in the preview image.
   - The image processing dialog box closes.
   - Please note that the image processing operation changes the source image. No new image document will be created. You can, however use the *Edit > Undo* command to restore the source image.
   - The changed image is not automatically saved. The fact that a change has to be saved will be indicated by an asterisk shown next to the image name in the document group.



The source image (**left**) has low contrast. Adjust the intensity to get significantly better contrast in the resulting image (**right**).

Cropping the image

8. In the *Smooth Control* tool window, click the *Crop to New Image* button.
9. With the left mouse button pressed, outline the image segment that interests you.
   - The segment you have selected will be identified by a red frame. The areas that are to be cut off are shaded.
   - In the *Smooth Control* tool window, the *Confirm Input* and *Cancel Input* buttons become active.

10. Confirm that this is the image segment you have selected. To do this, click the *Confirm Input* button.
    - A new image document called *Image_<serial No.>* is created.
    - The source image will not be changed.
11. In the *Smooth Control* tool window, click the *Close* button.
    - The *Smooth Control* tool window changes its appearance. The image acquisition functions are now shown again.

3.2.3. Discussing images

Task: You have acquired a number of images. Transfer the images to a colleague's computer using your intranet.

Prerequisite: The *NetCam* solution has been installed.

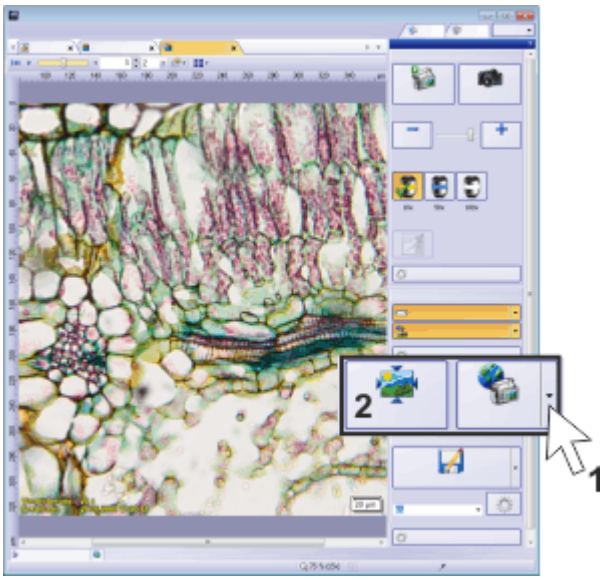
1. Load all of the images that you want to discuss.

Setting up a NetCam conference

2. In the *Smooth Control* tool window, click the small arrow next to the *NetCam* button. Select the *Settings* entry from the menu.

   - The *Options > NetCam > General* dialog box opens. On the left-hand side of the dialog box there is a tree view with all of the available options.
3. Select the *NetCam > Server* entry in the tree view.
4. Enter a password to make sure that only those persons who know the password have access to the images that are transferred via the NetCam client. Enter your password in the *Password* field.
5. In the *Server URL* field, you'll find the address of the server that the client needs for the web browser. Copy the NetCam server address.
6. Then close the *Options* dialog box with *OK*.
7. Send the NetCam server address and the password to all of your colleagues with whom you want to discuss the images.
8. In the *Smooth Control* tool window, click the *NetCam* button.

   - The button becomes active, thereby showing you that NetCam mode is active.
   - As long as NetCam mode is active, the contents of the image window are transferred to the NetCam server.
   - Your colleagues can now view the contents of the image window in their web browser when they have entered the NetCam address and authenticated themselves with the password. Please note that the field for the user name should remain empty for the authentication process.
9. Set up a telephone conference with your colleagues.



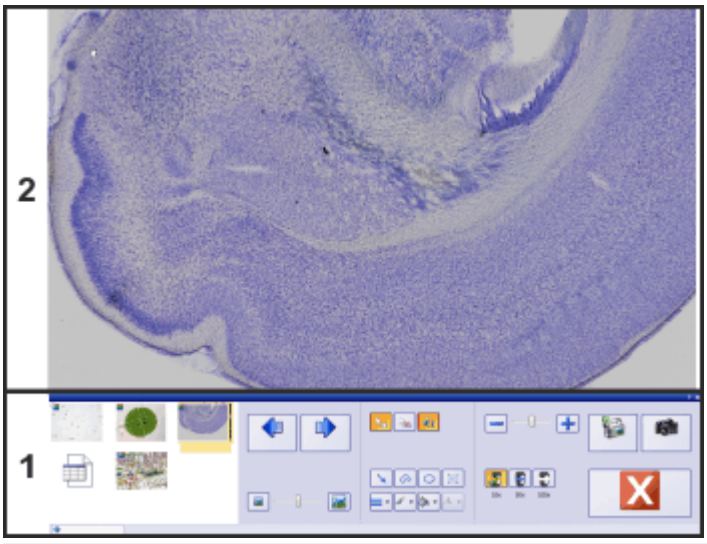
You can find the *Conference* group at the bottom of the *Smooth Control* tool window. Use the *NetCam* button (**1**) to check the NetCam settings and to switch to NetCam mode. Click the *Conference* button (**2**) to switch to conference mode.

Switching to conference mode

Note: If you don't use the NetCam function, you can also use the conference mode in the *Smooth Control* tool window to discuss your images using your monitor. All of the software controls on the user interface automatically scale to the resolution. They are also suitable for very high resolutions.

10. Click the *Conference* button to switch to conference mode.

- In conference mode your software automatically switches to full screen mode. The image will fill the whole monitor.
- The user interface is completely hidden except for the *Conference Mode* tool window.
- The *Conference Mode* tool window is minimized so that only its header is visible, at the bottom left of the monitor.
- The image in your colleagues' web browser will appear the same way. Your colleagues will also see any changes that you make in the image window.



In conference mode the image (**2**) fills the entire monitor. The *Conference Mode* tool window (**1**) is automatically hidden when you move the mouse pointer away from the tool window.

Drawing attention to a particular image segment

11. Display an image segment that particularly interests you in the image window.
    - Use the mouse wheel to zoom in to or out of the image.
    - If the entire image cannot be shown in the image window, you can move the image by dragging it while pressing the left mouse button.
12. Hover the mouse over the icon for the *Conference Mode* tab. You can find the icon at the bottom left-hand edge of the user interface.
    - The *Conference Mode* tool window opens.
    - The *Conference Mode* tool window isn't transferred by NetCam. NetCam transfers exclusively the contents of the image window.

13. Click the *Ellipse* button to circle the image segment that interests you. While pressing the left mouse button, draw an ellipse on the image.
    - Immediately after it has been inserted, the ellipse is selected. This enables you to change its properties straight away.
    - When you insert a drawing object, you automatically switch on drawing mode. The *Select Drawing Objects* button becomes active. You can recognize this status by the button's colored background.

14. You can change the color of the ellipse and select a thicker line width, for example, to make it easier to see in the image. To do this, use the *Line Width* and *Line Color* buttons.
    - NetCam transfers all of the drawing objects. This allows your colleagues to see which image segments you are discussing.
15. Close drawing mode. To do this, click the *Select Drawing Objects* button.

    - The *Select Drawing Objects* button now looks like this again.

Ending the conference

16. Click the *Close* button to leave conference mode.

- You now see the *Smooth Control* tool window, located on the right-hand side of the user interface again.

17. Release the *NetCam* button to stop transferring the images.
    - Then the *NetCam* button now looks like this again.

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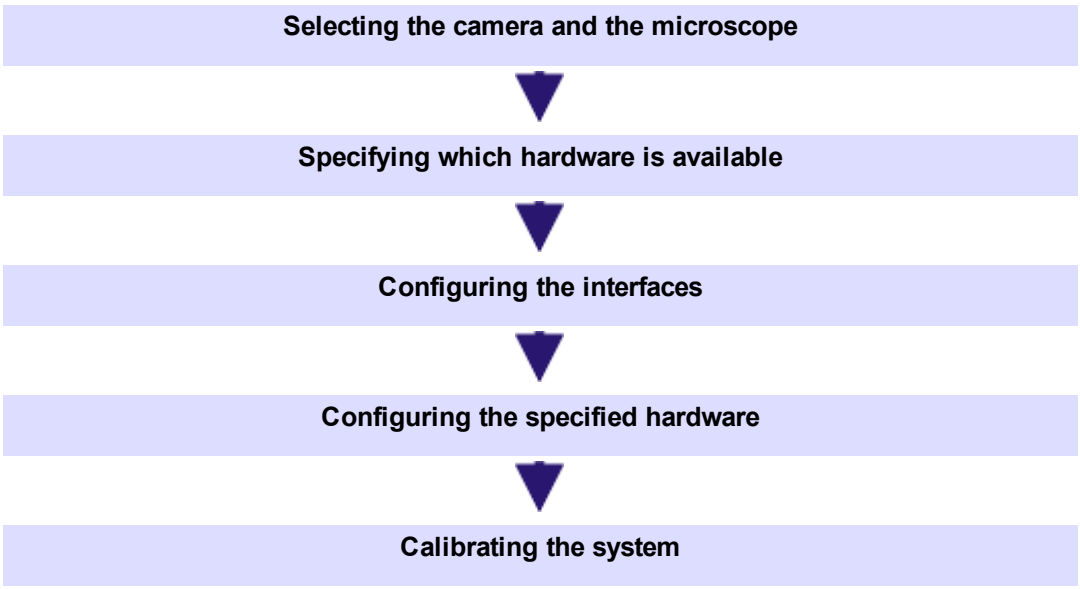
4. Overview - System Configuration

Why do you have to configure the system?

After successfully installing your software you will need to first configure your image analysis system, then calibrate it. Only when you have done this will you have made the preparations that are necessary to ensure that you will be able to acquire high quality images that are correctly calibrated. When you work with a motorized microscope, you will also need to configure the existing hardware, to enable the program to control the motorized parts of your microscope.

Process flow of the configuration

To set up your system, the following steps are necessary:



Selecting the camera and the microscope

The first time you start your software after the installation has been made, a quick configuration with some default settings will be made. In this step you need only to specify the camera and microscope types, in the *Quick Device Setup* dialog box. The microscope will be configured with a selection of typical hardware components.

Specifying which hardware is available

Your software has to know which hardware components your microscope is equipped with. Only these hardware components can be configured and subsequently controlled by the software. In the *Acquire > Devices > Device List* dialog box, you select the hardware components that are available on your microscope.

If you use a preset configuration that was offered in the *Quick Device Setup* dialog box, check now whether your system is really equipped with the hardware components that are defined in the configuration.

Configuring the interfaces

Use the *Acquire > Devices > Interfaces* command to configure the interfaces between your microscope or other motorized components, and the PC on which your software runs. Normally, the interfaces will automatically be configured properly.

If you use a preset configuration that was offered in the *Quick Device Setup* dialog box, you can skip this step.

Configuring the specified hardware

Usually, various different devices, such as a camera, a microscope and/or a stage will belong to your system. Use the *Acquire > Devices > Device Settings* dialog box to configure the connected devices so that they can be correctly actuated by your software.

Additionally, you can find all camera settings in the *Device Settings* dialog box.

Calibrating the system

When all of the hardware components have been registered with your software and have been configured, the functioning of the system is already ensured. However, it's only really easy to work with the system and to acquire top quality images, when you have calibrated your software. The detailed information that helps you to make optimal acquisitions will then be available.
Your software offers a wizard that will help you while you go through the individual calibration processes. Use the *Acquire > Calibrations* command to start the software wizard.

About the system configuration

When do you have to configure the system?

You will only need to completely configure and calibrate your system anew when you have installed the software on your PC for the first time, and then start it. When you later change the way your microscope is equipped, you will only need to change the configuration of certain hardware components, and possibly also recalibrate them.

Necessary user rights for the system configuration

To be able to configure the system, you have to be logged in to your software with administrator or power user rights. If you have installed the software yourself you will automatically have been assigned Administrator rights.

In contrast, other users that also want to work with the software are given the *User* role. The system configuration can't be changed or viewed by this role. The *Acquire > Devices > Device List* and *Acquire > Devices > Device Settings* commands are then no longer available.

For this reason, the software administrator has to assign the necessary user rights to those users who did not themselves install the software, but who are to be allowed to view or change the system configuration. Start the software as an administrator and select the *Tools > User Rights* command to open the *User Rights* dialog box. In it, select the required user, then click the *Properties* button.

Switching off your operating system's hibernation mode

Switch off your PC's power saving options and make sure that your PC does not automatically goes to hibernation mode.

1. To do so, right click the Start button located at the bottom left of the operating system's user interface.
2. Select the *Power options* entry from the menu.
3. Click *Sleep > Change plan settings* on the *Choose or customize a power plan* page.
4. On the *Change settings for the plan* page, click *Change advanced power settings*.
   - Here, you can switch off your PC's power saving and hibernation mode.

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5. Acquiring snapshots

You can use your software to acquire high resolution images in a very short period of time. For your first acquisition you should carry out these instructions step for step. Then, when you later make other acquisitions, you will notice that for similar types of sample many of the settings you made for the first acquisition can be adopted without change.

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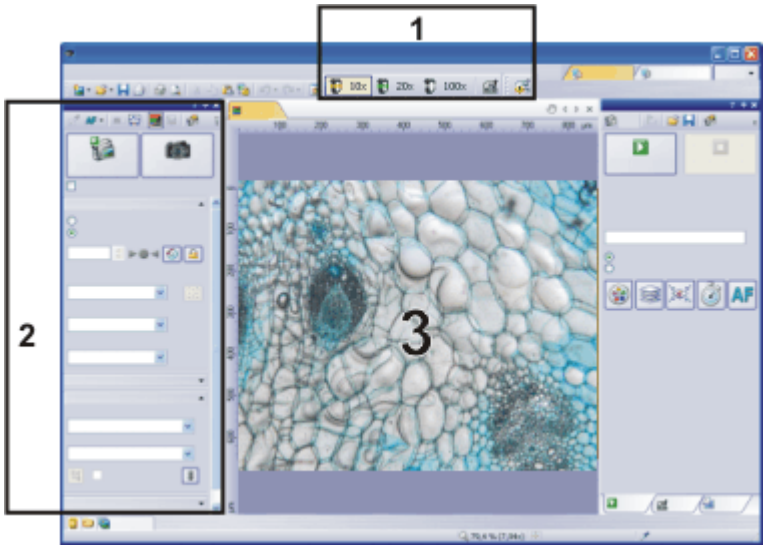
5.1. Acquiring a snapshot

1. Switch to the *Acquisition* layout. To do this, you can use the *View > Layout > Acquisition* command.
   - You can find the *Microscope Control* (**1**) toolbar at the upper edge of the user interface, right below the menu bar.
     To the left of the document group, you see the *Camera Control* (**2**) tool window.



Selecting an objective

2. On the *Microscope Control* toolbar, click the button with the objective that you use for the image acquisition.

Switching on the live-image

3. In the *Camera Control* tool window, click the *Live* button.
   - The live-image (**3**) will now be shown in the document group.
4. Go to the required sample position in the live-image.

Setting the image quality

5. Bring the sample into focus. The *Focus Indicator* toolbar is there for you to use when you are focusing on your sample.

Note: For some cameras, the *Focus Peaking* function is available to help you to focus on your sample.

6. Check the color reproduction. If necessary, carry out a white balance.
7. Check the exposure time. You can either use the automatic exposure time function, or enter the exposure time manually.
8. Select the resolution you want.

Acquiring and saving an image

9. In the *Camera Control* tool window, click the *Snapshot* button.
   - The acquired image is shown in the document group.
10. Use the *File > Save As* command to save the image. Use the recommended TIF or VSI file format.

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5.2. Changing what the live window does

The live-image will get its own new window in the software's document group. This window's title will be *Live (active)*. What this live window does when live-mode stops depends on the settings in the *Live* group. What the live window does depends on the acquisition settings in the *Acquisition Settings > Acquisition > General* dialog box.

Continuing the live mode after the image has been acquired

1. Make the *Camera Control* tool window appear. To do this, you can use the *View > Tool Windows > Camera Control* command.
2. Open the *Acquisition Settings > Acquisition > General* dialog box.

   To do so, click the *Acquisition Settings* button in the *Camera Control* tool window's toolbar.

   Select the *Acquisition > General* option in the tree view.
3. Select the *Continue live after snapshot* check box.
4. Close the *Acquisition Settings* dialog box with *OK*.

5. Click the *Live* button in the *Camera Control* tool window.
   - A temporary live window called *Live (active)* will be created in the document group.
   - The live-image will be shown in the live window.

   - You can always recognize the live mode by the changed look of the *Live* button in the *Camera Control* tool window.

6. Click the *Snapshot* button to acquire a snapshot.
   - A new image window for the image that has been acquired appears in the document group. The image that is acquired is called *Image_<consecutive number>* by default.
   - The software automatically goes back to live-mode.
   - The live-image is displayed in the document group.
7. Acquire additional images.

8. Click the *Live* button again to leave live mode.
   - The live mode will be switched off.

Switching the live mode on and off without acquiring an image

1. Select the following settings in the *Acquisition Settings > Acquisition > General* dialog box.
   - Choose the *Keep document when live is stopped* option.
   - Clear the *Create new document when live is started* check box.
   - Clear the *Continue live after snapshot* check box.

2. Click the *Live* button in the *Camera Control* tool window.
   - A temporary live window called *Live (active)* will be created in the document group.
   - The live-image will be shown in the live window.

   - You can always recognize the live mode by the changed look of the *Live* button in the *Camera Control* tool window.

3. Click the *Live* button again.
   - The live mode will be switched off.
   - The active live image will be stopped.
   - The live window's header will change to *Live (stopped)*. You can save the stopped live-image located in the live window just as you can every other image.

Note: The live window may look similar to an image window, but it will be handled differently. The next time you switch on the live mode, the image will be overwritten. Additionally, it will be closed without a warning message when your software is closed.

Switching to the live mode and acquiring an image

1. Select the following settings in the *Acquisition Settings > Acquisition > General* dialog box.
   - Choose the *Keep document when live is stopped* option.
   - Clear the *Create new document when live is started* check box.
   - Clear the *Continue live after snapshot* check box.

2. Click the *Live* button in the *Camera Control* tool window.
   - A temporary live window called *Live (active)* will be created in the document group.
   - The live-image will be shown in the live window.

   - You can always recognize the live mode by the changed look of the *Live* button in the *Camera Control* tool window.

3. Click the *Snapshot* button.
   - The live mode will be switched off. The live window's header will change to *Live (stopped)*.
   - At the same time, a new image document will be created and displayed in the document group. You can rename and save this image. If you have not already saved it when you end your software, you will be asked if you want to do so.

Displaying the live-image and the acquired images simultaneously

Task: You want to view the live-image and the acquired images simultaneously. When you do this, it should also be possible to look through the acquired images without having to end the live mode.

1. Close all open documents.
2. Open the *Acquisition Settings > Acquisition > General* dialog box.

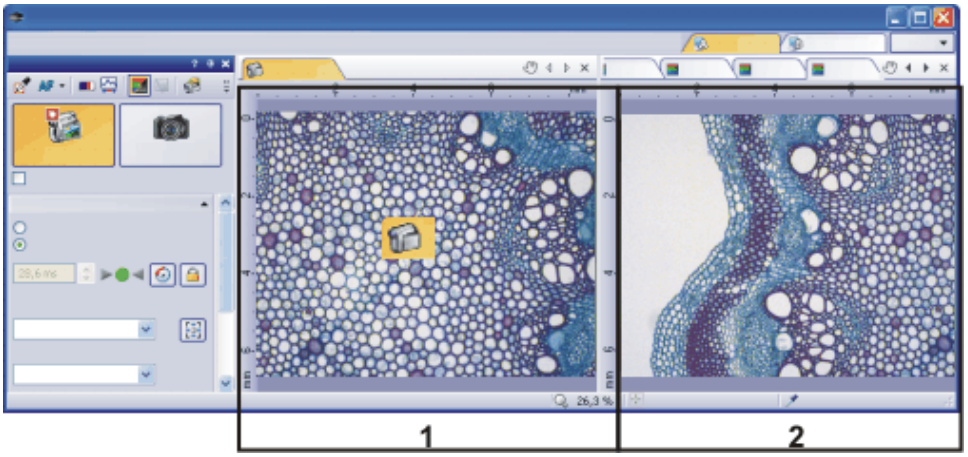
   To do so, click, for example, the *Acquisition Settings* button on the *Camera Control* tool window.
3. There, make the following settings:
   - Choose the *Keep document when live is stopped* option.
   - Clear the *Create new document when live is started* check box.
   - Select the *Continue live after snapshot* check box.
4. Switch to the live mode. Acquire an image, then switch the live mode off again
   - Both of the image windows *Live (stopped)* and *Image_<consecutive number>* are now in the document group.
   - The *Live (stopped)* image window is active. That's to say, right now you see the stopped live-image in the document group. In the document bar, the name *Live (stopped)* is highlighted.
5. Split the document group, to have two images displayed next to each other.

   That's only possible when at least two images have been loaded. That's why you created two images in the first step.

   To do this, use the *Window > Split/Unsplit > Split/Unsplit Document Group (Left)* command.
   - This command creates a new document group to the left of the current document group. In the newly set up document group the active document will be automatically displayed. Since in this case, the active document is the stopped live-image, you will now see the live window on the left and the acquired image on the right.

6. Start the live mode.
   - In the document group, the left window will become the live window *Live (active)*. Here, you see the live-image.
7. Activate the document group on the right. To do so, click, for example, the image displayed there.

8. Click the *Snapshot* button.
   - The acquired image will be displayed in the active document group. In this case, it's the document group on the right.
   - After the image acquisition has been made, the live-image will automatically start once more, so that you'll then see the live-image again on the left.
   - While the live-image is displayed on the left, you can switch as often as you want between the images that have already been acquired.



You can set up your software's user interface in such a way that you can view the live-image (**1**) and the images that have up till then been acquired (**2**), next to one another.

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5.3. Acquiring HDR images

HDR stands for **H**igh **D**ynamic **R**ange. Dynamic range relates to the capacity of cameras or software to display both bright and dark image segments.

Before acquiring an HDR image, the necessary exposure range needs to be determined for the current sample. The exposure range is made up of a minimum and maximum exposure time as well as several exposure times between them. A recently determined exposure range will continue to be used for all HDR images until you let your software determine the exposure range anew. It is irrelevant whether the exposure range had been determined automatically or manually.

If you are acquiring several images of the same or similar parts of a sample, you don't need to determine the exposure range each time. If you change the sample or adjust settings on the microscope, it is recommended to determine the exposure range anew (either automatically or manually).

Acquiring an HDR image with a manually determined exposure range

With this procedure, you set the minimum and maximum exposure time in the *Camera Control* tool window yourself. Your software guides you through the process with relevant message boxes. How much the exposure time is adjusted by is determined by your software with regards to the minimum and maximum exposure time.

Preparations

1. Switch to the *Acquisition* layout. To do this, use the *View > Layout > Acquisition* command, for example.
2. On the *Microscope Control* toolbar, click the button with the objective that you want to use for the acquisition of the HDR image.
3. Switch to live mode and select optimal settings for your acquisition, in the *Camera Control* tool window. Carry out a white balance. Choose an approximate exposure time.
4. Search for the part of the sample which you want to acquire an HDR image of. This should be a position which has such significant differences in brightness that not all segments can be shown with optimal lighting.
5. Finish the live mode.

Acquiring HDR image

6. In the *Camera Control* tool window, select the *Enable HDR* check box.
   - In the upper part of the tool window, the *Snapshot* button changes to the *HDR* button.

   
7. In the *Determine exposure range* group, click the *Manual* button to define the exposure range for this acquisition anew.
   - The *Determine exposure range* message box appears. It prompts you to reduce the exposure time so far that enough image details can be recognized in the bright image segments and no segments are overexposed.
8. Change the exposure time in the *Exposure* group, which is part of the *Camera Control* tool window. Make sure that the *Manual* option is chosen. You can change the value by using the slide control or by entering an exposure time with the keyboard and pressing the [Enter] key. Check the live image on display. Once the bright image segments are no longer overexposed, click the *OK* button in the *Determine exposure range* message box.
   - By doing so, you have determined the lower limit of the exposure range (= the shortest exposure time).
9. Now, the *Determine exposure range* message box prompts you to raise the exposure time so high that the dark image segments are no longer underexposed. Change the exposure time in the *Exposure* group, which is part of the *Camera Control* tool window. Check the live image on display. Once the dark image segments are bright enough, click the *OK* button in the *Determine exposure range* message box.

- By doing so, you have determined the upper limit of the exposure range (= the longest exposure time).

10. Click the *HDR* button in the *Camera Control* tool window to start the image acquisition.
    - The image acquisition will begin. Pay attention to the progress bar located in the status bar 

. It shows how long the acquisition has taken and the total acquisition time. The progress bar contains the *Cancel* button, which you can use to stop the current image acquisition.
    - After the acquisition has been completed the HDR image will be shown in the document group.
11. Check the image. If you want to change the settings (to use a different algorithm for the output rendering, for example), open the *Acquisition Settings* dialog box. Select the *Acquisition > HDR* entry in the tree view.
12. If you don't want to change any settings, use the *File > Save As* command to save the image. Use the recommended TIF or VSI file format.
    - These are the only formats which also save all the image information including the HDR entries together with the image. In this way, you can see, e.g., whether or not an image was acquired using HDR. Open the *Properties* tool window, and look at the data in the *Camera* group.

Acquiring more HDR images without setting the exposure range anew

If you have just acquired HDR images of the same or a similar sample, as a rule, it is not necessary to determine the dynamic range anew. In this case, you have already completed the preparations for acquisition (such as carrying out a white balance) and set the HDR image acquisition settings correctly (such as choosing the optimal algorithm used for output rendering) anyway.

In such circumstances, acquiring an HDR image is especially easy. Do the following:

1. In the *Camera Control* tool window, select the *Enable HDR* check box.
2. Click the *HDR* button in the *Camera Control* tool window to start the image acquisition.
    - The image acquisition will begin. After the acquisition has been completed the HDR image will be shown in the document group.
3. Check the image before saving it.
    - This step can be left out if your software is configured to import images into a database directly after acquisition.

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5.4. Working with the Super Resolution System

What is a Super Resolution System?

The Super Resolution System is composed of particular hardware components and software functions that can be used to create super resolution images.

Prerequisites:

- The Olympus Super Resolution (OSR) functions are available when the *Super Resolution* Solution is active.
- The super resolution hardware components are configured and the software is installed.

The software offers a variety of different super resolution filters which can be used to compute a super resolution image. A super resolution filter can either be applied when a fluorescence image is being acquired or at a later point in time. In both cases, the optical and technical requirements for the acquisition of a super resolution image must be fulfilled.

Note: Before working with the Super Resolution System, make absolutely sure that the lasers are connected in accordance with the applicable safety regulations.

Hardware requirements

The following hardware components and settings are required for the creation of super resolution images:

- Microscope (IX83 P2ZF)
- Confocal Scanner Unit (CSU-W1(T1) or CSU-W1(T2))
- Camera (Hamamatsu Orca Flash 4.0 v3)
- Motorized magnification changer (1x magnification, 3.2x magnification)
- Laser Combiner
- Real Time Controller (RTC-E)

Objective, magnification factor, and binning

To create super resolution images, certain lenses are suitable that can be combined with binning and magnification by a magnification changer.

Disk changer

If a disk changer with multiple spinning disk types is configured on your system, the following combinations are possible for creating super-resolution images.

Objective magnification	Motorized magnification changer	Spinning disk	Binning
100x	3,2x	50µm or SoRa	2x2
60x	3,2x	50µm or SoRa	1x1

Laser wave lengths

The following laser wavelengths are suitable for the creation of super resolution images.

Excitation (nm)	405	445	488	514	561	640

5.4.1. Configuring the Super Resolution System

The following process flow chart displays the basic steps of the process.

Defining the hardware configuration
In the *Device List* dialog box, enter all of your system's hardware components.

▼

Defining an observation method

Defining the hardware configuration

1. Select the *Acquire > Devices > Device List* command.
2. Select the *Camera* tab.
3. Select the camera from the *Camera 1* list.
   If you are working with a multi camera system, select the *Multi Camera #1* entry from the corresponding *Multi Camera* list. If you are working with an additional camera, select the second camera from the *Camera 2* list. Select the *Multi Camera #2* entry from the corresponding *Multi camera* list.
4. Select the *Confocal* tab.
5. Select the CSU (Confocal Scanner Unit) from the *System* list.
6. If the CSU is equipped with an aperture stop, select the *Variable aperture control* check box.
7. Select the *Lasers/LEDs* tab.
8. Select the *Laser Combiner* entry in the *Device* list.

9. Select the *OBIS Laser* entry in the *Type* list. Expand the list with the + button and add all of the lasers that are to be coupled to the fiber.
10. Click the *Interfaces* button and configure the CSU interface in the *Interfaces* dialog box.
11. Close all open dialog boxes.

Defining an observation method

Certain optical requirements have to be fulfilled before your software can compute a super resolution image. Define an observation method for super resolution images in which you employ a super resolution filter and specify the system components that super resolution requires.

1. Select the *Acquire > Devices > Device Customization* command. Activate the *Observation Methods* tab.
2. Click the *New Observation Method* button.
3. Give the new observation method a name, then close the dialog box with *OK*. You can call the observation method *Super Resolution*, for example.
4. Select the *Camera > <camera name>* entry in the *Available components* list. If you are using a multi camera system, select the *Camera > Multi camera (<camera name>)* entry in the *Available components* list.
5. Select the *Use* entry from the *Status* list.
6. If you are using a multi camera system with two cameras, also select the *Multi camera configuration* check box.
7. In the *Available components* list, select the *Super Resolution* entry for each camera.
8. For each camera, select the *Use* entry in the *Status* list.
   - The *OSR filter* list is now active.
9. Select a super resolution filter for each camera from the *OSR filter* list. If you are using two cameras, you can select different filters for them.
   - The *Low*, *Standard* and *High* filters refer to the strength of the super resolution filter. Which filter is most suitable for your sample depends on the properties of the sample. Try out the different filters and select the filter with the best results.
   - Use the *Offline* entry if you don't want to apply the super resolution filter straight when the image is acquired, but at a later point in time. This can make sense if you are performing an experiment on a sensitive sample with the Experiment Manager and you want to keep the experiment as short as possible. When the *Offline* entry is active, the images are acquired with the settings required for super resolution images. You can retroactively apply a super resolution filter to the images that have been acquired using the *Filter: Super Resolution* function.
   - When an observation method with an OSR filter is used, the software checks whether the requirement for super resolution images are fulfilled before making an acquisition (live, snapshot, acquisition process). If the conditions are not met, the acquisition is not started.

10. You can now assign a fluorochrome and a fluorescence color to the cameras. This makes sure that the acquired images are automatically displayed in the right color.

Note: To create super resolution images, certain lenses are suitable that can be combined with binning and magnification by a magnification changer.

11. Define the magnification factor you want for the observation method in the *Available components* > *General* > *Magnification Changer* list. Alternatively, you can set a magnification factor in the *Microscope Control* tool window.
12. For the observation method, define any additional components that you are working with.

5.4.2. Acquiring super resolution images

1. In the *Microscope Control* tool window, select the observation method with the settings for super resolution images.
2. Select the required objective.

Note: To create super resolution images, certain lenses are suitable that can be combined with binning and magnification by a magnification changer.

3. Choose the binning value you want in the *Resolution* group located in the *Camera Control* tool window.
4. In the *Camera Control* tool window, click the *Live* button or the *Snapshot* button.
   - The software checks whether the settings fulfill the requirements for super resolution images. If the requirements for super resolution images are not fulfilled, an error message appears.

Note: If the images of the individual fluorescence color channels are misaligned by a few pixels, you can correct this using the *Multi Channel Shift Correction* calibration process.

5. You can automatically add the OSR filter that was used to create the super resolution image to the image name. Use the *All Options* dialog box to add the <OSR Filter> placeholder to the image name.

5.4.3. Acquiring super resolution images with the Experiment Manager

Use the Experiment Manager to acquire super resolution images in an experiment.

1. Use the *View > Tool Windows > Experiment Manager* command to display the *Experiment Manager* tool window.
2.  In the *Experiment Manager* tool window, click the *New* button to create a new experiment.
3.  Define the first image acquisition command.
   Click the small arrow next to the *Image Acquisition* button to open a menu. Select the observation method that was defined for super resolution images, *Super Resolution* for example.

4. Click the canvas at the position where you want to place the image acquisition command with the *Super Resolution* observation method in the experiment plan.
5. If necessary, define further commands.
6.  Click the *Start* button, located in the *Experiment Manager* tool window to run the experiment.
   - The software checks whether the settings fulfill the requirements for super resolution images.
   - If these requirements are not fulfilled, an error message appears telling you which parameters are missing.

5.4.4. Deconvolution filters for super resolution images

Use the *Process > Deconvolution > Constrained Iterative* command and select the *Super Resolution* modality to apply a deconvolution filter to super resolution images. This filter can remove diffused light to improve super resolution images.

Prerequisites:

- The *Constrained Iterative* deconvolution filter is available when the *CI Deconvolution* software solution is active.
- The *Constrained Iterative* deconvolution filter can only be applied to Z-stacks and time stack-Z-stacks.

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6. Multi-dimensional image

What is a multi-dimensional image?

You can combine a series of individual images into one image. You can, e.g., assemble separate images that belong to different color channels. Depending on how the frames differ, the multi-dimensional image that results from their combination will also vary.

A standard image is two dimensional. The position of every pixel will be determined by its X- and Y-values. Fluorescence color, time and Z-position of the microscope stage are the possible additional dimensions of a multi-dimensional image.

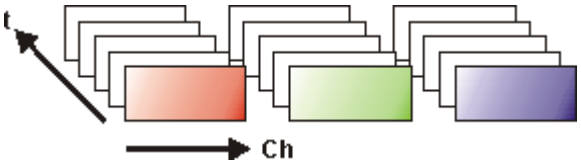
A multi-channel image as a rule shows a sample that has been marked with several different fluorochromes. The multi-channel image is made up of a combination of the individual fluorescence images.

In a time stack all frames have been acquired at different points of time. A time stack shows you how an area of a sample changes with time. You can play back a time stack just as you do a movie.

A Z-stack contains frames, which belong to different focus positions. You need a Z-stack, for instance, for the calculation of an EFI image.

Image containing several dimensions

The different multi-dimensional images can be arbitrarily combined. A multi-channel time stack, for example, incorporates several color channels. Every color channel incorporated in the image is reproduced with its own time stack.



Navigation bar in the image window

The multi-dimensional images have their own navigation bar directly in the image window. Use this navigation bar to define how a multi-dimensional image is to be displayed in the image window.

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6.1. Overview - Acquisition processes

Your software offers a wide range of different acquisition processes.

Note: Your software is available in a variety of versions. This help contains the description of all acquisition processes. For this reason, it can happen that your software version doesn't have some of the acquisition processes described here.

Basic acquisition processes

Complex acquisition processes

Combination of several acquisition processes

6.1.1. Basic acquisition processes

Use the *Camera Control* tool window to acquire images and movies.

Acquisition process - Snapshot

You can use your software to acquire high resolution images in a very short period of time.

Acquisition process - Movie

You can use your software to record a movie. When you do this, your camera will acquire as many images as it can within an arbitrary period of time. The movie can be saved as a file in the AVI or VSI format. You can use your software to play it back.

6.1.2. Complex acquisition processes

Use the *Process Manager* tool window to handle complex acquisition processes.

Acquisition process - Time Lapse

With the automatic acquisition process *Time Lapse* you acquire a series of frames one after the other. This series of individual images makes up a time stack. A time stack shows you how an area of a sample changes with time. You can play back a time stack just as you do a movie.

You can combine the *Time Lapse* acquisition process with other acquisition processes. If your software supports the *Multi Channel* acquisition process, use, e.g., the *Time Lapse* acquisition process to acquire a multi-channel time stack.

If your microscope stage is equipped with a motorized Z-drive, when you acquire a time stack you can use the autofocus. You can find a description of the individual settings along with the description of the acquisition process.

Acquisition process - Z-stack

Use the automatic acquisition process *Z-Stack* to acquire a Z-stack. A Z-stack contains frames acquired at different focus positions. That is to say, the microscope stage was located in a different Z-position for the acquisition of each frame.

Alternatively, you can also acquire an EFI image with the *Z-Stack* acquisition process. Then a resulting image (EFI image) with a practically unlimited depth of focus is automatically calculated from the Z-stack that has been acquired. Such an image is focused throughout all of its segments. EFI is the abbreviation for Extended Focal Imaging.

You can combine the *Z-Stack* acquisition process with other acquisition processes. If your software supports the *Multi Channel* acquisition process, you can combine the *Z-Stack* acquisition process with the multi channel acquisition to acquire a multi-channel Z-stack.

Acquisition process - XY-Positions/MIA

You can only use this acquisition process when your microscope is equipped with a motorized XY-stage. With this acquisition process you can carry out one or more automatic acquisition processes at different positions on the sample or acquire a stitched image of a larger sample position.

If your microscope stage is equipped with a motorized Z-drive, you can use the autofocus for this acquisition process. You can find a description of the individual settings along with the description of the acquisition process.

Acquisition process - Multi Channel

With the automatic acquisition process *Multi Channel* you acquire a multi-channel fluorescence image.

You can combine, e.g., the *Multi Channel* acquisition process with the *Z-stack* acquisition process to acquire a multi-channel Z-stack.

Note: When you use the DP80 camera, please note the following restriction. When you acquire a transmitted light image simultaneously with a multi-channel fluorescence image, the *Multi Channel* acquisition process can't be combined with another acquisition process, for example the *Z-stack* acquisition process. This restriction protects the camera from being damaged by permanently toggling between the two CCDs the camera provides.

Acquisition process - Instant EFI

Use the manual acquisition process *Instant EFI* to acquire an EFI image at the camera's current position that is sharply focused all over.

Acquisition process - Manual MIA

When you use the *Manual MIA* acquisition process, you move the stage manually in such a way that different, adjoining sample areas are shown. Every time you click one of the buttons with an arrow, an image is acquired. With this acquisition process, you combine all of the images that are acquired, directly during the acquisition, just like a puzzle, into a stitched image. The stitched image will display a large sample segment in a higher XY-resolution than would be possible with a single acquisition.

Acquisition process - Instant MIA

For the *Instant MIA* acquisition process, you slowly move the stage manually over all of the positions on the sample that you want to acquire for the MIA image. Your software acquires images continuously and automatically assembles them. You just have to start the acquisition process, the acquisition of the individual images takes place automatically as you move the stage.

6.1.3. Combination of several acquisition processes

You can combine several automatic acquisition processes. To do so, click the corresponding button for each acquisition process you require.

Note: Which automatic acquisition processes you can combine with each other, depends on your software.

Examples

When you combine the two acquisition processes *Multi Channel* and *Z-Stack*, a complete multi-channel image will be acquired at every focus position. You can specify the order of the acquisition processes in the [*C*] group in the *Process Manager* tool window. You define the acquisition parameters for the *Multi Channel* acquisition process in this group.

When you combine the two acquisition processes *Z-Stack* and *XY-Positions/MIA* to acquire a Z-stack at several positions on your sample, to begin with, the complete Z-stack at the first position will be acquired. When that has been done, your system will move to the next position, and will acquire the next Z-stack etc..

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7. Acquiring image series

You can use your software to acquire images series. Images series can be time stacks or Z-stacks.

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7.1. Time stacks

What is a time stack?

You can combine a series of individual images into one image. In a time stack all frames have been acquired at different points of time. A time stack shows you how an area of a sample changes with time. You can play back a time stack just as you do a movie.

A standard image is two dimensional. The position of every pixel will be determined by its X- and Y-values. With a time stack, the time when the image was acquired is an additional piece of information or dimension for each frame.

The frames making up a time stack can be 8-bit gray-value images, 16-bit gray-value images or 24-bit true-color images.

Note: A time stack can also be an AVI video. You can load and play back the AVI file format with your software.

How do I recognize a time stack?

You can immediately recognize the different image types by the icon which appears in front of the image name in the document group, or in the *Documents* tool window. When it is a time stack, this icon will be supplemented by a small clock. A time stack that is made up of true-color images has, for example, this icon  .

In the *Properties* tool window, you can use the *Frame Count* entry to find out how many frames are contained in any given image.

A time stack will automatically have its own navigation bar directly in the image window. Use this navigation bar to browse through the frames making up a time stack, or to play back the time stack like a movie.

Creating time stacks

There are different ways in which you can generate a time stack.

To acquire a time stack, use one of the two acquisition processes *Time Lapse* or *Movie*.

Use the *Image > Combine Frames* command to have several individual images combined into a time stack.

Displaying time stacks

A time stack contains much more data than can be displayed on your monitor.

A time stack will automatically have its own navigation bar directly in the image window. Use this navigation bar to determine which of the frames from a time stack is to be displayed on your monitor. You can also play back a time stack just as you would a movie.

Alternatively, you can also use the *Dimension Selector* tool window to determine how a time stack is to be displayed on your monitor, or to change this.

Hiding the navigation bar

You can also hide the navigation bar. To do this, use the *Tools > Options* command. Select the *Images > General* entry in the tree view. Clear the *Show image navigation toolbar* check box.

Saving time stacks

When you save time stacks, you will, as a rule, use the VSI file format. Only when you use this file format is there no limit to the size a time stack can be. When you save smaller time stacks, you can also use the TIF or the AVI file format. With any other file format you will lose most of the image information during saving. To do so, use the *File > Save As* command.

Converting time stacks

Breaking down time stacks into individual images

Use the *Image > Separate > Time Frames* menu command to have a time stack broken down into selected individual images.

Reducing the number of frames within a time stack

It is possible that, within a time stack, only a short period of time interests you. Use the *Extract* command to create a new time stack that only contains a selection of frames, from an existing time stack. In this way, you will reduce the number of frames within a time stack to only those that interest you. You will find this command in the context menu in the tile view for time stacks.

Converting time stacks while saving them

When you save a time stack in another file format as TIF or VSI, the time stack will also be converted. The time stack will then be turned into a standard true-color image. This image shows the frame that is at that moment displayed on the monitor.

Processing time stacks

Image processing operations, for example, a sharpen filter, affect either the whole image or only a selection of individual images. You will find most of the image processing operations in the *Process* menu.

The dialog box that is opened when you use an image processing operation has the same structure for every operation. In this dialog box, select the *Apply on > Selected*

frames and channels option to determine that the function only affects the selected frames.

Select the *Apply on > All frames and channels* option to process all of the individual images.

Select the individual images that you want to process, in the tile view. Look through the thumbnails and select the images you want to process. In the tile view, the standard MS-Windows conventions for multiple selection are valid.

An image processing operation does not change the source image's dimensions. The resulting image is, therefore, comprised of the same number of separate images as the source image.

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7.2. Time Lapse / Movie

Both the *Time Lapse* and the *Movie* acquisition processes document the way a sample changes with time. What is the difference between the two processes?

When is it better for me to acquire a time stack?

Use the *Time Lapse* acquisition process in the following cases:

- Use the *Time Lapse* acquisition process when processes that run slowly are to be documented, for example, where an acquisition is to be made only every 15 minutes.
- Use the *Time Lapse* acquisition process when, while the acquisition is in progress, you want to see the frames that have already been acquired, for example, to check on how an experiment is progressing. To do this, click the *Tile View* button in the navigation bar in the image window.
- Use the *Time Lapse* acquisition process when you want to use those of your software's additional functions that can only be saved in the VSI or TIF file format. For example, to measure objects, to insert drawing elements such as arrows, or a text, or to have the acquisition parameters for the camera and microscope that you've used, available at any time in the future.
- Use the *Time Lapse* acquisition process when the important thing is to achieve an optimal image quality, and the size of the file is no problem.

Saving a time stack as an AVI

You can also save a time stack as an AVI file, at a later date. To do this, load the time stack into the document group, select the *File > Save As* command and select the AVI file type. Make, if necessary, additional settings in the *Select AVI Save Options* dialog box.

When is it better for me to acquire a movie?

Use the *Movie* acquisition process in the following cases:

- Use the *Movie* acquisition process when processes that run very quickly are to be documented (the number of acquisitions per second is considerably higher with movies than with time stacks).
- Use the *Movie* acquisition process when you want to give the movie to third persons who do not have this software (AVI files can also be played back with the MS Media Player).
- Use the *Movie* acquisition process when keeping file sizes small is of great importance.

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7.3. Acquiring movies and time stacks

With your software you can acquire movies and time stacks.

Acquiring a movie

You can use your software to record a movie. When you do this, your camera will acquire as many images as it can within an arbitrary period of time.

1. Switch to the *Acquisition* layout. To do this, use the *View > Layout > Acquisition* command, for example.

Setting the magnification

2. On the *Microscope Control* toolbar, click the button with the objective that you want to use for the movie acquisition.

   If you are using a magnification changer, you will also have to select the magnification value used.

Selecting the storage location

3. In the *Camera Control* tool window's toolbar, click the *Acquisition Settings* button.
   - The *Acquisition Settings* dialog box opens.
4. Select the *Saving > Movie* entry in the tree structure.
5. You have to decide how a movie is to be saved after the acquisition. Select the *File system* entry in the *Automatic save > Destination* list to automatically save the movies you have acquired.
   - The *Path* field located in the *Directory* group shows the directory that will currently be used when your movies are automatically saved.
6. Click the [...] button next to the *Path* field to alter the directory.
7. In the *File type* list, select the file format in which you want to save the movie. You can save the movie either as a VSI image or as an AVI video. You can select the *AVI Video File (*.avi)* entry.

Selecting the compression method

8. Click the *Options* button when you want to compress the AVI file in order to reduce the movie's file size.
9. Select, for example, the *Motion JPEG* entry from the *Encoder* list.

Select the *Medium* entry in the *Quality* list.

Close the *Movie Options* dialog box with *OK*.

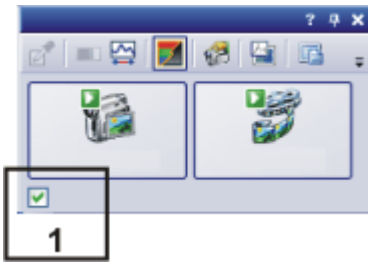
10. Close the *Acquisition Settings* dialog box with *OK*.

Setting the image quality

11. Switch to the live-mode and select the optimal settings for movie acquisition in the *Camera Control* tool window. Pay special attention to setting the correct exposure time.
    - This exposure time will not be changed during the movie recording. Even if you have set the exposure time to automatic, the exposure time won't be adjusted while the movie is being recorded.
12. Find the segment of the sample that interests you and focus on it.

Switching to movie recording mode

13. Select the *Movie recording* check box (**1**). The check box can be found below the *Live* button in the *Camera Control* tool window.



- The *Snapshot* button will be replaced by the *Movie* button.

Starting movie recording

14. Click the *Movie* button to start the movie recording.
    - The live-image will be shown and the recording of the movie will start immediately.
    - In the status bar a progress indicator is displayed. At the left of the slash the number of already acquired images will be indicated. At the right of the slash an estimation of the maximum possible number of images will be shown. This number depends on your camera's image size and cannot exceed 2 GB.

    - This icon on the *Movie* button will indicate that a movie is being recorded at the moment.

Stopping movie recording

15. Click the *Movie* button again to end the movie recording.
    - The first image of the movie will be displayed.
    - The navigation bar for time stacks will be shown in the document group. Use this navigation bar to play the movie.
    - The software will remain in the movie recording mode until you clear the *Movie recording* check box.

Acquiring a time stack

In a time stack all frames have been acquired at different points of time. With a time stack you can document the way the position on the sample changes with time. To begin with, for the acquisition of a time stack make the same settings in the *Camera Control* tool window as you do for the acquisition of a snapshot. Additionally, in the *Process Manager* tool window, you have to define the time sequence in which the images are to be acquired.

Task: You want to acquire a time stack over a period of 10 seconds. One image is to be acquired every second.

1. Switch to the *Acquisition* layout. To do this, use the *View > Layout > Acquisition* command, for example.

Setting the magnification

2. On the *Microscope Control* toolbar, click the button with the objective that you want to use for the movie acquisition.
   If you are using a magnification changer, you will also have to select the magnification value used.

Setting the image quality

3. Switch to the live mode, and select the optimal settings for your acquisition, in the *Camera Control* tool window. Pay special attention to setting the correct exposure time. This exposure time will be used for all of the frames in the time stack.
4. Choose the resolution you want for the time stack's frames, from the *Resolution > Snapshot/Process* list.
5. Find the segment of the sample that interests you and focus on it.

Selecting the acquisition process

6. Activate the *Process Manager* tool window.
7. Select the *Automatic Processes* option.

8. Click the *Time Lapse* button.
    - The button will appear clicked. You can recognize this status by the button's colored background.
    - The [*t*] group will be automatically displayed in the tool window.
9. Should another acquisition process be active, for example, *Z-Stack*, click the button to switch off the acquisition process.
    - The group with the various acquisition processes could, for example, now look

like this:

Setting the acquisition parameters

10. Clear the check boxes *Start delay* and *As fast as possible*.
11. Specify the time that the complete acquisition is to take, e.g., 10 seconds. Enter the value 00000:00:10,000 in the *Recording time* field, to set the recording time to 10 seconds. You can directly edit every number in the field. To do so, simply click in front of the number you want to edit.
12. Click the button with the lock located to the right of the field to specify that the acquisition time is no longer to be changed.
13. Specify how many frames are to be acquired.
    Enter e.g., 10 in the *Cycles* field.
    - The *Interval* field will be updated. It shows you the time that will elapse between two consecutive frames.

Acquiring a time stack

14. Click the *Start* button.
    - The acquisition of the time stack will start immediately.
    - The *Start* button changes into the *Pause* button. A click on this button will interrupt the acquisition process.
    - The *Stop* button will become active. A click on this button will stop the acquisition process. The images of the time stack acquired until this moment will be preserved.
    - At the bottom left, in the status bar, the progress bar will appear. It indicates the number of images that are still to be acquired.
    - The acquisition has been completed when you can once more see the *Start* button in the *Process Manager* tool window, and the progress bar is no longer displayed.
    - You will see the time stack you've acquired in the image window. Use the navigation bar located in the image window to view the time stack.
    - By default, the time stack that has been acquired will be saved automatically. The storage directory is shown in the *Acquisition Settings > Saving > Process Manager* dialog box. The preset file format is VSI.

Note: When other programs are running on your PC, for instance a virus scanning program, it can interfere with the performance when a time stack is being acquired.

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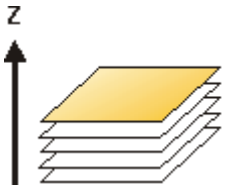
7.4. Z-stack

What is a Z-stack?



You can combine a series of separate images into one image file. A Z-stack contains frames acquired at different focus positions. A Z-stack is needed, e.g., for calculating an EFI image by the *Process > Enhancements > EFI Processing* command.

A standard image is two dimensional. The position of every pixel will be determined by its X- and Y-values. With a Z-stack, the focus position or the height of the sample is an additional item of information for every pixel.

The frames making up a Z-stack can be 8-bit gray-value images, 16-bit-gray-value images or 24-bit-true-color images.

How do I recognize a Z-stack?

You can immediately recognize a multi-dimensional image by its icon which appears in front of the image name in the document group or in the *Documents* tool window. When it is a Z-stack, this icon will be supplemented by a small Z .

In the *Properties* tool window, you can use the *Frame Count* entry to find out how many frames are contained in any given image.

A Z-stack image will automatically have its own navigation bar directly in the image window. Use this navigation bar to browse through the frames making up a Z-stack, or to play back the Z-stack like a movie.

Creating a Z-stack

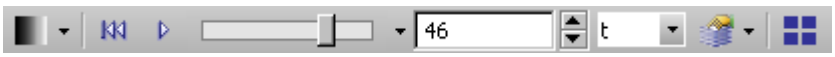
There are different ways in which you can generate a Z-stack.

1. To acquire a Z-stack, use the *Z-Stack* acquisition process.
2. Use the *Image > Combine Frames* command to have several separate images combined into a Z-stack.

Displaying a Z-stack

A Z-stack contains much more data than can be displayed on your monitor.

A Z-stack image will automatically have its own navigation bar directly in the image window. Use this navigation bar to determine which of the frames from a Z-stack is to be displayed on your monitor. You can also play back the Z-stack just as you would a movie.



Alternatively, you can also use the *Dimension Selector* tool window to define how a Z-stack image is to be displayed on your monitor, or to change this.

Saving a Z-stack

Please note: Z-stacks can only be saved in the TIF or VSI file format. Otherwise they lose a great deal of their image information during saving.

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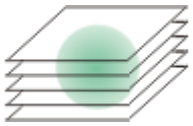
7.5. Acquiring Z-stacks

A Z-stack contains frames acquired at different focus positions. That is to say, the microscope stage was located in a different Z-position for the acquisition of each frame.

Note: You can only use the *Z-Stack* acquisition process when your stage is equipped with a motorized Z-drive.

Task: You want to acquire a Z-stack. The sample is approximately 50 µm thick. The Z-distance between two frames is to be 2 µm.



1. Switch to the *Acquisition* layout. To do this, use, e.g., the *View > Layout > Acquisition* command.

Selecting an objective

2. On the *Microscope Control* toolbar, click the button with the objective that you want to use for the image acquisition.

Setting the image quality

3. Switch to the live mode, and select the optimal settings for your acquisition, in the *Camera Control* tool window. Pay special attention to setting the correct exposure time. This exposure time will be used for all of the frames in the Z-stack.
4. Search out the required position in the sample.

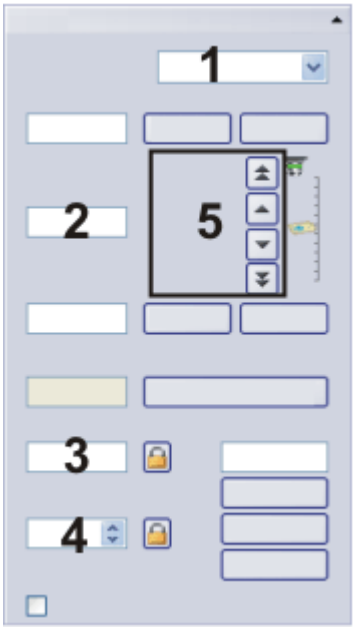
Selecting the acquisition process

5. Activate the *Process Manager* tool window.
6. Select the *Automatic Processes* option.

7. Click the *Z-Stack* button.
   - The button will appear clicked. You can recognize this status by the button's colored background.
   - The [*Z*] group will be automatically displayed in the tool window.
8. Should another acquisition process be active, e.g., *Multi Channel*, click the button to switch off the acquisition process.

- The group with the various acquisition processes should now look like this:

Setting the acquisition parameters



Set the acquisition parameters for the acquisition of a Z-stack in the *Process Manager* tool window. Use the fields and buttons (**1**-**4**) for this.

9. Select the *Range* entry in the *Define* list (**1**).
10. Enter the Z-range you want, in the *Range* field (**2**). In this example, enter a little more than the sample's thickness (= 50 µm), e.g., the value 60.
11. In the *Step Size* field (**3**), enter the required Z-distance, e.g., the value 2, for a Z-distance of 2 µm. The value should roughly correspond to your objective's depth of focus.
    - In the *Z-Slices* field (**4**) you will then be shown how many frames are to be acquired. In this example 31 frames will be acquired.
12. Find the segment of the sample that interests you and focus on it. To do this, use the arrow buttons (**5**). The buttons with a double arrow move the stage in larger steps.

Acquiring an image

13. Click the *Start* button.
    - Your software now moves the Z-drive of the microscope stage to the start position. The starting positions lies half of the Z-range deeper than the stage's current Z-position.
    - The acquisition of the Z-stack will begin as soon as the starting position has been reached. The microscope stage moves upwards step by step and acquires an image at each new Z-position.

- The acquisition has been completed when you can see the *Start* button in the *Process Manager* tool window again, and the progress bar is no longer displayed.
- You can see the acquired Z-stack in the image window. Use the navigation bar located in the image window to view the Z-stack.
- The Z-stack that has been acquired will be automatically saved. You can set the storage directory in the *Acquisition Settings* > *Saving* > *Process/Experiment* dialog box. The preset file format is VSI.

Note: When other programs are running in the background on your PC, for instance a virus scanning program, it can interfere with the performance when a Z-stack is being acquired.

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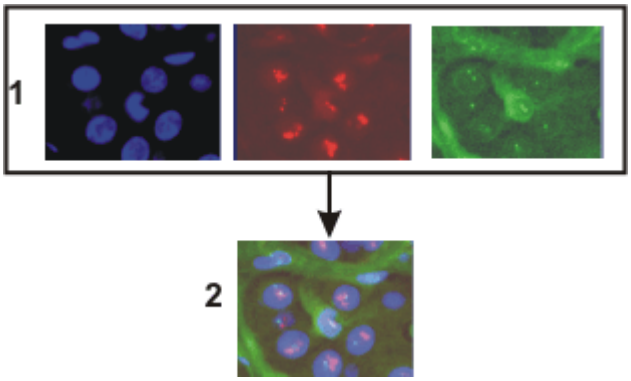
8. Acquiring and editing fluorescence images

8.1. Multi-channel image

What is a multi-channel image?

A multi-channel image combines a series of monochrome images into one image. The multi-channel image usually shows a sample that has been stained with several different fluorochromes. The multi-channel image is made up of a combination of the individual fluorescence images. You can have the individual fluorescence images displayed separately or also as a superimposition of all of the fluorescence images.



At the top of the illustration you can see the individual fluorescence images (**1**).
Below, you can see the superimposition (**2**) of the separate fluorescence images.

The separate images making up a multi-channel image can be 8-bit gray-value images or 16-bit gray-value images.

A multi-channel image can be combined with a time stack or a Z-stack. A multi-channel time stack incorporates several color channels in that case. Every color channel incorporated in the image is reproduced with its own time stack.

How do I recognize a multi-channel image?

You can immediately recognize a multi-channel image by this icon which appears in front of the image name in the document group or in the *Documents* tool window.

In the *Properties* tool window, you can use the *Channels* entry to find out how many channels are contained in any given image.

A multi-channel image will automatically have its own navigation bar, directly in the image window. Use this navigation bar to set how a multi-channel image is to be displayed in the image window, or to change this.

Creating multi-channel images

Your software offers you several ways of acquiring a multi-channel image.

1. Use the *Multi Channel* automatic acquisition process to acquire a multi-channel image.

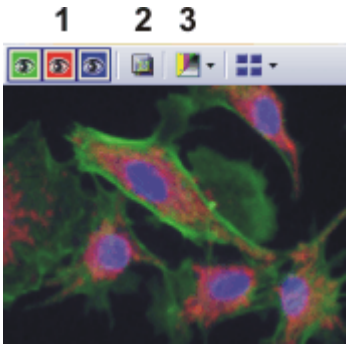
2. Use the Experiment Manager to define and run complex experiments involving image acquisition with your software. Use the *Multichannel Group* command to assemble images into a multi-dimensional image.
3. Use the *Image > Combine Channels* command to have several separate images combined into a multi-channel image.

Special hardware for acquiring multi-channel fluorescence images

Your software supports both image splitters and multi camera systems. Both systems enable you to acquire more than one color channel simultaneously, and to assemble them into a multi-channel fluorescence image.

Displaying multi-channel images

A multi-channel image contains much more data than can be displayed on your monitor.



When you load a multi-channel image into your software, you'll see a navigation bar in the image window, that provides you with access to all of the fluorescence channels.

- You can have each fluorescence image displayed separately or also as a superimposition of all of the fluorescence images (**1**).
- Should you have acquired a brightfield of the sample together with the fluorescence images, you can make this brightfield appear or disappear (**2**).
- The individual fluorescence images are monochrome. For this reason, you can change the color mapping however you like. You can display the fluorescence channels in the fluorescence colors, use a pseudo color table of your choice, or also display the source images (**3**).

Hiding the navigation bar

You can also hide the navigation bar. To do this, use the *Tools > Options* command. Select the *Images > General* entry in the tree view. Clear the *Show image navigation toolbar* check box.

Using the Dimension Selector

Alternatively, you can also use the *Dimension Selector* tool window to define how a multi-channel image is to be displayed on your monitor, or to change this. There you can, for example, change the fluorescence colors for individual color channels.

Saving multi-channel images

Please note: Multi-channel images can only be saved in the TIF or VSI file format. Otherwise they lose a great deal of their image information during saving.

Converting multi-channel images

Breaking down a multi-channel image into its color channels

Use the *Separate* command to have a multi-channel image broken down into chosen color channels. The resulting images are still of the "multi-channel" type, contain though, only one color channel.

There are several ways of accessing this command:

- Click the *Separate Channels* button in the *Dimension Selector* tool window.
- Use the *Separate* command from the *Dimension Selector* tool window's context menu.
- Use the *Image > Separate > Channels* menu command.

Reducing the color channels in a multi-channel image

Use the *Extract* command to create a new multi-channel image that is made up of fewer color channels than the source image.

Select all of the color channels you wish to retain, in the *Dimension Selector* tool window. Then use the *Extract* command in the tool window's context menu.

Converting multi-channel images while saving them

When you save a multi-channel image in another file format as TIF or VSI, the multi-channel image will also be converted. The multi-channel image then becomes a standard 24-bit true-color image. This image will always show exactly what is currently displayed on your monitor, that is to say, for example, the superimposition of all of the channels or possibly only one channel.

Processing multi-channel images

Image processing operations, for example, a sharpen filter, affect either the whole image or only a selection of individual images. You will find most of the image processing operations in the *Process* menu.

Select the frames that you want to process in the *Dimension Selector* tool window. The channels you have selected will be highlighted in color in the tool window.

The dialog box that is opened when you use an image processing operation has the same structure for every operation. In this dialog box, select the *Apply on > Selected frames and channels* option to determine that the function only affects the selected frames.

Select the *Apply on > All frames and channels* option to process all of the individual images.

An image processing operation does not change the source image's dimensions. The resulting image is, therefore, comprised of the same number of separate images as the source image.

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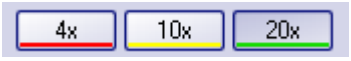
8.2. Before and after you've acquired a fluorescence image

8.2.1. Before you acquire a fluorescence image

Defining observation methods

1. Define observation methods for your color channels.

Setting up the microscope for the acquisition of a fluorescence image

1. Use the *View > Tool Windows > Microscope Control* command to make the *Microscope Control* tool window appear.
   - In the *Objectives* group, you can find the buttons you use to change objectives.
     

   - In the *Observation method* group you can find a button for every observation method that has been defined. Observation methods should have been defined at least for brightfield and for every color channel.
2. Click the required objective's button.
3. Click the button for the observation method with the excitation that has the longest excitation wavelength (e.g. Red) .

Setting up the camera for the acquisition of a fluorescence image

1. Use the *View > Tool Windows > Camera Control* command to make the *Camera Control* tool window appear.
2. Set the image resolution for the acquisition. With a high objective magnification, you require a lower resolution.
   For this purpose, select the required resolution from the *Snapshot/Process* list, located in the *Resolution* group.
3. Reduce the image resolution in the live mode. When you use a higher binning in the live mode, the frame rate will be reduced, which enables you to focus better.
   For this purpose, select an entry, e.g., with the supplement *Binning 2x2*, from the *Live/movie* list, located in the *Resolution* group.
4. Should you work with a color camera: Switch on your camera's grayscale mode.
   You can find this button on the *Camera Control* tool window's toolbar.

- The appearance of the *Toggle RGB/Grayscale mode* button has changed.

5. If it's possible to set different bit depths with your camera, click the *Toggle bit depth* button to set the maximum bit depth.

Switching off the corrections for brightfield acquisitions

1. Use the *View > Toolbars > Calibrations* command to have the *Calibrations* toolbar displayed.

2. Switch off the white balance and the shading correction.
   To do so, release these buttons , if they are there and available.

Focusing a fluorescence sample

1. Select the automatic exposure time.

2. In the *Camera Control* tool window, click the *Live* button.
   - Should the live-image be too dark, select a higher exposure compensation value. To do so, use the *Camera Control > Exposure > Exposure compensation* slide control.
   - Should the exposure time become longer than 300 ms, reduce it by increasing the sensitivity or gain.
3. Bring the sample into focus.

   - In the camera's black & white mode, you can reduce the diffused light. Click the *Online-Deblur* button, located in the *Camera Control* tool window's toolbar. You can then decide whether or not you want to apply the deconvolution filter. It's possible that you may have to increase the exposure time by using the exposure compensation.

4. Finish the live mode. To do so, click the *Live* button in the *Camera Control* tool window.

Setting the storage location

Multi-channel images will be saved by default, as soon as the acquisition has been completed. As file format, the VSI file format will be used.

1. Before you start the acquisition, specify where the file is to be saved.

2. To do this, click the *Acquisition Settings* button, located in the *Process Manager* tool window's toolbar. Select the *Saving > Process Manager* entry in the tree view.
   - You can find the current directory in the *Directory > Path* field.
3. Click the [...] button next to the *Path* field to change the directory into which the image is to be saved after its acquisition.

8.2.2. After you've acquired a fluorescence image

Viewing a multi-channel image

A multi-channel image is made up of the individual fluorescence images. You can set which color channels, or combination of color channels, will be displayed on your monitor. To do this, use the navigation bar in the image window.

Click a color field to make the channel appear or disappear. All of the color channels that are at the moment displayed on your monitor will be identified by an eye icon.

The navigation bar also offers you additional possibilities for changing the appearance of the multi-channel image.

Viewing the acquisition parameters

Numerous acquisition parameters are saved together with the image.

Use the *View > Tool Windows > Properties* command to make the *Properties* tool window appear. In the *Properties* tool window, you can find that every color channel has its own *Channel* information group. This contains the channel name, the emission wavelength, the name of the observation method and the exposure time.

Saving multi-channel images

The multi-channel image will be automatically saved. You can set the storage directory in the *Acquisition Settings > Saving > Process Manager* dialog box. The file format used is VSI.

For the VSI format, a JPEG compression of 90% is preset. You can change the compression in the *Acquisition Settings* dialog box under *Saving > Process Manager > Automatic save > Options…* .

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8.3. Defining observation methods for the fluorescence acquisition

Before you acquire a fluorescence image, you have to define observation methods for your color channels. Usually, observation methods that you can adapt for your microscope configuration, have already been predefined.

Prerequisite

The system has already been configured and calibrated. For this purpose, you have to enter your hardware components in the *Acquire > Devices > Device List* dialog box, and configure them in the *Device Settings* dialog box. To finalize this action, calibrate your system by using the *Acquire > Calibrations* command.

The tables that follow, contain example configurations for both motorized, and not motorized microscopes. Only the hardware components that are relevant for the acquisition of multi-channel fluorescence images are listed.

Device List		Example entries	
		Non-motorized microscope	**Motorized microscope**
Microscope Frame	<Name of your microscope>	BX51	BX61
Microscope			
Nosepiece	<Name of your nosepiece>	Manual Nosepiece	Motorized (UCB)
Mirror turret	<Name of your mirror turret>	Manual mirror turret	BX-RFAA
Stage			
Z-axis	<Name of your controller for the stage's Z-drive>	Not Motorized	Motorized (UCB)
Fluorescence/reflected light path			
Shutters	<Name of the reflected light shutter>	Manual Shutter	Motorized (UCB)
Transmission light path			
Lamp	<Name of your transmission lamp>	Not used	UCB Halogen-Lamp
Condenser	<Name of your condenser>	Manual Condenser	U-UCD8A

Device Settings	Entries
Nosepiece	<Your objectives>
Mirror turret	U-MNU U-MWB U-MWG For a position where there is no mirror cube, select the *Free* entry.
Condenser	With a motorized condenser: The hardware components *Aperture Stop* and *Top Lens* are additionally listed under the device settings.

8.3.1. Defining the observation method for transmission brightfield

Example: Hardware components in transmission brightfield: In transmission brightfield, there is no fluorescence mirror cube in the light path. The reflected light shutter is closed.

1. Use the *Acquire > Devices > Device Customization* command. Activate the *Observation Methods* tab.
2. Click the *New Observation Method* button.
   - A dialog box, in which you can enter a name, will be opened.
3. Give the new observation method a name, then close the dialog box with *OK*. You could name your observation method, e.g., MyBF.

Setting the mirror turret

4. Select the mirror turret in the *Available components* list.
   - In the central area of the dialog box, the settings for the hardware components that have been selected will be displayed.
5. In transmission brightfield, the mirror turret isn't allowed to contain a mirror cube. Therefore, select the *Adjust* entry in the *Status* list, located in the middle of the dialog box, and in the list that's below that one select the *Free* entry.
   - For manual microscopes: Where a manual microscope is concerned, the mirror turret can't be automatically moved to the position you want. When you use a manual microscope, a message appears prompting you to make this setting manually.
     This message will also appear when you are defining the observation method. Confirm the message with *OK*.

For motorized microscopes: Setting up the condenser

6. Select the condenser.

   Select the *Adjust* entry in the *Status* list, located in the middle of the dialog box, and in the list that's below that one, the *Free* entry.

7. Select the hardware component *Aperture Stop*.

   Select the *Adjust* entry in the *Status* list, located in the middle of the dialog box. Enter "75%" in the field below that.
8. Select the hardware component *Top Lens*.
   Select the *Adjust per objective* entry in the *Status* list.
   - In the middle of the *Device Customization* dialog box, you can now set the top lens for each objective separately.
   - The top lens is only used for objectives with higher magnifications (upwards of 10x) and is swung out for lower magnifications.
9. Specify for which objectives the top lens should be brought into the light path and for which objectives it should be removed from the light path.
   - To do so, select the *Use with this objective* check box for all objectives.
   - In the *Selected components* list, each objective with a lower magnification than 10x needs to show the *Out* status. If that isn't the case, click in the middle of the dialog box on the *Out* button.
   - In the *Selected components* list, each objective with a higher magnification than 10x or exactly 10x needs to show the *In* status. If that isn't the case, click in the middle of the dialog box on the *In* button.

For motorized microscopes: Switching on the transmission lamp

10. Select the transmission lamp. You will find this lamp in the *Available components* list, under the *Transmission* entry.
11. Select the *Adjust* entry in the *Status* list, located in the middle of the dialog box. Set 9 V for the lamp.

    Use the button showing a small lamp to switch on the lamp.
    - The button looks like this, if the lamp is switched on.

      
    - In the *Selected components* list, you can also see that the lamp for this observation method will be switched on.

Saving the observation method

12. Click the *OK* button to save the new observation method.
    - The *Microscope Control* tool window will then contain a new button with this observation method's name.
    - You can now use the observation method in the *Process Manager* tool window, for the acquisition of a multi-channel fluorescence image.

8.3.2. Defining observation methods for fluorescence channels

Task: Define an observation method for the acquisition of a fluorescence image. It also makes sense to do this when you don't work with a motorized microscope, since then the acquired image will be automatically colored with the correct fluorescence color.

The following hardware components belong to an observation method for fluorescence channels. How you integrate these hardware components with the observation method, is described in detail in these step-by-step instructions.

Hardware components	Settings
Fluorochromes	Assign the fluorescence colors.
Mirror turret	Choose the mirror cube to be used.
Fluorescence shutter	Open the fluorescence shutter for the image acquisition.
Transmitted light lamp	Switch off the transmitted light lamp.

1. Use the *Acquire > Devices > Device Customization* command. Activate the *Observation Methods* tab.
2. Click the *New Observation Method* button.
3. Give the new observation method a name, then close the dialog box with *OK*. Name the observation method, e.g., Blue.

Defining a fluorochrome

4. Assign the fluorescence channel a fluorochrome (e.g., DAPI) and a color (e.g., Blue at 470 nm). To do so, select the *Fluorochromes* entry in the *Available components* list.

   Select the *Use* entry in the *Status* list.

   In the *Fluorochrome* list located below that one, select the fluorochrome to be used, e.g., the entry *Blue* or *DAPI*.

   You can change the fluorescence color in the *Color* list, should that be necessary.

   - It is important in all cases to define the fluorochrome for a fluorescence observation method, even if you don't automatically change any device settings. When the fluorescence color is linked to the observation method, every image you acquire with this observation method will be automatically colored in the corresponding color. This is valid irrespective of whether you work with a manual or a motorized microscope.
   - Note: Don't select the *No Color* entry in the *Fluorochrome* list. If you do this, the observation method won't be recognized as a fluorescence observation method in the *Process Manager* tool window.

For motorized microscopes: Setting the mirror turret

It can make sense to use this setting and the additional settings for motorized microscopes also for manual microscopes. When you use a manual microscope, a message appears prompting you to make this setting manually. As well as this, the device settings are saved together with the acquired image.

5. Select the mirror turret in the *Available components* list.
   - In the central area of the dialog box, the settings for the hardware components that have been selected will be displayed.
6. For the acquisition of a fluorescence image a specific mirror cube has to be used. Therefore, select the *Adjust* entry in the *Status* list, located in the middle of the dialog box, and in the list that's below that one, select the mirror cube you want.

For motorized microscopes: Setting the shutter for the fluorescence light path

7. In the *Available components* list, select the shutter that is located below the *Fluorescence/reflected* entry.
8. When the fluorescence acquisition is made, this shutter must be open. Therefore, select the *Use for acquisition* entry in the *Status* list, located in the middle of the dialog box.
   - Then the shutter will be opened before the image acquisition is made, and closed when this has been done, to avoid bleaching of the sample.

For motorized microscopes: Switching off the transmission lamp

9. Select the transmission lamp. You will find this lamp in the *Available components* list, under the *Transmission* entry.
10. For the acquisition of a fluorescence image, the lamp must be switched off. Select the *Adjust* entry in the *Status* list, located in the middle of the dialog box.

    Use the button showing a small lamp to switch off the lamp.
    - The button looks like this, if the lamp is switched off.

      
    - In the *Selected components* list, you can also see that the lamp for this observation method will be switched off.

Including camera settings

It's usually better to use a black & white camera for acquiring fluorescence images. If you are using a camera that can toggle between a color mode and a grayscale mode, you can integrate the grayscale mode into the observation method.

This setting is not necessary if you acquire fluorescence images with the *Multi Channel* acquisition process. Before the *Multi Channel* acquisition process starts, your software checks whether or not your camera is working in the grayscale mode. You will then receive a corresponding message, and can reset the camera before the image acquisition is made.

11. Select your camera in the *Available components* list.
12. Select the *Use* entry in the *Status* list.
13. Some cameras offer grayscale modes in different bit depths. Select the grayscale mode with the highest bit depth from the *Image type* list.

Saving the observation method

14. Click the *OK* button to save the new observation method.
    - The *Microscope Control* tool window will then contain a new button with this observation method's name.
    - You can now use this color channel for the *Multi Channel* acquisition process.

8.3.3. Using predefined observation methods

As a rule you don't have to completely redefine the observation method required. Use one of the predefined observation methods, and customize it for your microscope.

1. Use the *Acquire > Devices > Device Customization* command. Activate the *Observation Methods* tab.
    - In the *Name* list, you will find all of the observation methods that have been predefined.

      Should no observation methods be available, click the *Select Predefined Observation Methods*  button. Click the *Select All* button. Click *OK*.
    - As soon as mirror cubes have been entered for the mirror turret, in your device settings, the appropriate observation methods will be automatically set up. You will always find the observation method beneath the mirror cube's name.
2. Select a fluorescence channel (e.g., U-MNU) in the *Name* list.
3. Click the *Rename Observation Method* button.
    - The *Enter a New Observation Method Name* dialog box opens.
4. Enter a more general name (e.g., Blue or DAPI), then click *OK*.
    - The *Selected components* list contains the following hardware components. There can be more or fewer components, depending on what you have chosen in the way of hardware components in the device list.
    - In the mirror turret, the corresponding mirror cube (e.g., U-MNU) will be selected.
    - The transmission lamp will be switched off.
    - The transmission light path's shutter will have the *Use for acquisition* status. This means that it will be open when the image is acquired.
5. Assign a fluorochrome (such as DAPI) to the fluorescence channel.
6. Click the *OK* button to save the new observation method.
    - The *Microscope Control* tool window will then contain a new button with this observation method's name.

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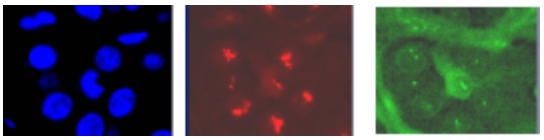
8.4. Acquiring a fluorescence image

Your software supports several image types. The multi-channel image usually shows a sample that has been stained with several different fluorochromes. However, it is also possible to acquire a multi-channel image that consists only of one single channel.



The illustration shows three fluorescence images of the same sample position. Each image shows another fluorochrome.

Switching to a dark user interface

1. If you are disturbed by the light of your monitor, you can switch your software to a dark user interface. To do so, use the *View > Dark Application Skin* command.

Selecting the fluorescence observation method

2. Use the *View > Toolbars > Observations Methods* command to have the *Observation Methods* toolbar displayed.
3. To load an observation method, click the button with the name of the fluorescence observation method you want.
   - For manual microscopes: Loading a fluorescence observation method defines that a fluorescence image is to be acquired. For your software, all observation methods using the *Fluorochromes* component are automatically identified as fluorescence observation methods.
   - For motorized microscopes: When you load an observation method, this leads to the microscope being brought into a defined condition. To do so, all of the microscope's motorized components will be brought into exactly the position that has been defined for these components in the observation method.

Selecting the exposure time for the acquisition

4. Use the *View > Tool Windows > Camera Control* command to make the *Camera Control* tool window appear.

5. Switch to the live mode. To do so, click the *Live* button in the *Camera Control* tool window.
   - For motorized microscopes: The reflected light shutter will be automatically opened.

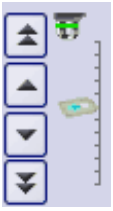
     This behavior will be specified when the observation method is defined. For the shutter, the *Use for acquisition* status should have been selected.
6. In the *Camera Control* tool window, select the *Exposure > Manual* option.
7. Some cameras offer the *SFL* mode for fluorescence acquisitions (for example, the DP74). Switch this mode on.
8. Optimize the exposure time.

   Should the exposure time become longer than 500 ms, reduce it by increasing gain.

To do this, change the value in the *Exposure > Sensitivity* field or use the *Gain* slide control.

Focusing a fluorescence sample

9. Bring the image into focus.
   - If your microscope stage is equipped with a motorized Z-drive, a focus regulator will be at your disposal in the *Microscope Control* tool window.



10. Finish the live mode. To do so, click the *Live* button in the *Camera Control* tool window.
    - For motorized microscopes: The reflected light shutter will be closed.
    - In the image window, you will now see the fluorescence image that has been acquired. The fluorescence image has the image type "Multi-channel image" even if it consists only of one single channel.

      You can immediately recognize a multi-channel image by this icon which appears in front of the image name in the document group or in the *Documents* tool window.
    - The fluorescence image will be displayed using the fluorescence color that you have defined together with the observation method.

Changing the fluorescence image's display

11. You can use the *Dimension Selector* tool window to define how the fluorescence image is to be displayed on your monitor, or to change this. There you can, for example, change the fluorescence color.

Saving the fluorescence image

12. Use the *File > Save As* command afterwards to save the new multi-channel image. Use the TIF file format.

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8.5. Combine Channels

The *Image > Combine Channels* command creates a new multi-channel image from several separate images.

Which images can you combine?

Gray-value images

You can combine a series of gray-value images into a multi-channel image. These can be either 8-bit gray-value images or 16-bit gray-value images. The prerequisite therefore, is that all of the separate images have the same bit depth, image size and image calibration.

Multi-channel images

Multi-channel images don't necessarily have to be made up of several color channels. There can also be multi-channel images that only contain one fluorescence channel. You can also combine these images into a new multi-channel image that then contains several fluorescence channels. The prerequisite therefore, is that all of the separate images have the same bit depth, image size and image calibration.

Multi-dimensional images

You can combine several multi-dimensional images into one image. Prerequisite for such an operation is that all of the individual images only differ in one dimension (color channel, focus position, or time point), and are of the same image size.

One example of this is two single color time stacks that are each made up of 50 separate images. Each time stack was acquired with a different color channel. In this case you can create one multi-channel time stack.

Transmitted light images

Sometimes another image that shows the same position on the sample in the transmitted light mode, belongs to a series of fluorescence images. You can combine such a transmitted light image with the multi-channel image.

The transmitted light image doesn't have to be of the same image type as the separate images. However, the image size, image calibration, and the bit depth have to be the same as the fluorescence images.

For example: You can use a true-color image as a transmitted light image. When the individual fluorescence images have a bit depth of 16 bit, you can only use a 48-bit true-color image as a transmitted light image.

8.5.1. Combining fluorescence images

1. Load the gray-value images that you want to combine into a multi channel fluorescence image. The sample was, for example, marked with the fluorochromes, DAPI and Texas Red.
2. Activate the first image in your software's document group.

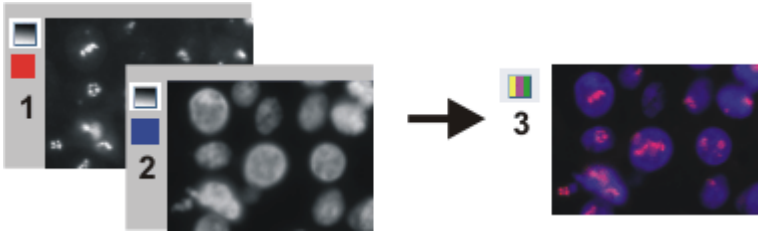
Combining channels

3. Use the *Image > Combine Channels* command.
   - The *Combine Channels* dialog box opens.
   - In the *Available Images* table, the active image will be automatically entered as the first color channel.
   - When you have acquired the individual fluorescence images with a suitable observation method, the name of the color channel and the fluorescence color will be read out of the image and automatically used in the *Combine Channels* dialog box.
4. In case you have to change the channel name or want to do so: Click once in the *Name* cell. Enter a description of the color channel, for example, the name of the fluorochrome used "Texas Red".

   You can increase the width of the row so that the description will fit into it.
5. When the fluorescence color can't be read out of the image, the first channel will, by default, be assigned the color "Red". To change the active color, click this color field. Select one of the colors from the palette on the *Standard* tab, or activate the *Custom* tab to define a color of your choice.

   Click the *OK* button, to close the color palette and return to the *Combine Channels* dialog box.
6. In the next free row, click the *Images* cell. You will be presented with a picklist containing all of the images that you can combine with the active image. Select your next image. As soon as you click in another row, the new image will be included in the sheet.
7. You can now change the characteristics of the new channel. Give the new channel a name and assign a color.
8. It is possible to shift the individual images with respect to each other. To do this, if necessary, use the arrow buttons in the *Pixel shift* group.
9. You can set the weighting of the individual color channels. Increase, for example, the value in the *Intensity* field to weight a channel more strongly.
10. Click the *OK* button to create your multi-channel image.

    A new image document with the default name "Image_<serial No.>" is created.



With the *Combine Channels* command, you combine fluorescence images (**1**) and (**2**) into a multi-channel image (**3**), this can be done with more than two images also.

Saving a multi-channel image

11. Use the *File > Save As* command to save your new multi-channel image. Always use the TIF or VSI file format when saving an image.

Viewing a multi-channel image

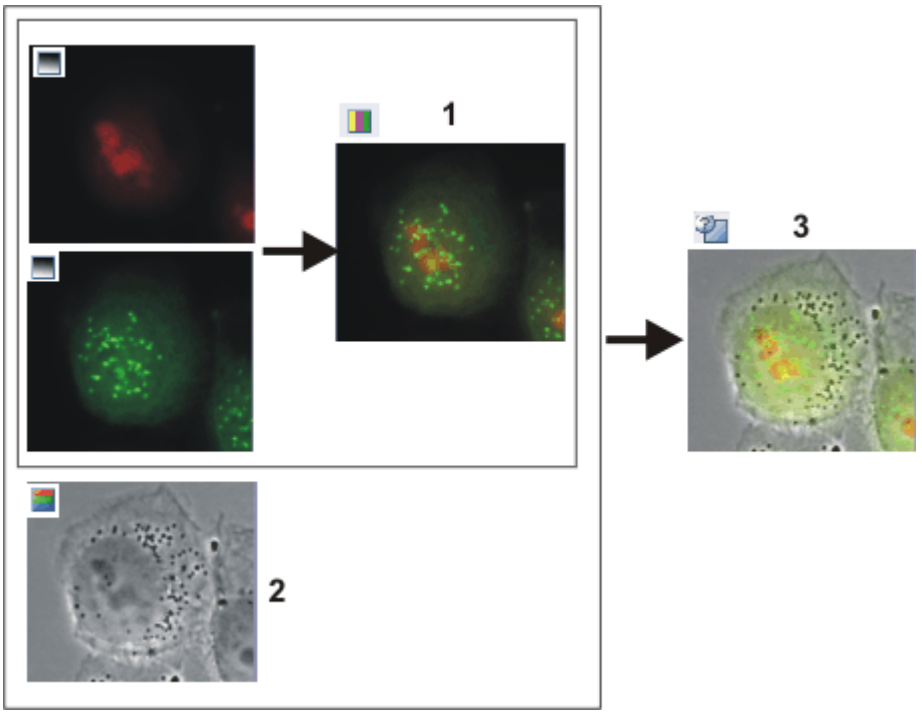
12. Use the tool window to change the fluorescence color, to choose another color mapping, or to switch individual color channels off and back on.
13. Use the *Adjust Display* tool window to change the display of a multi-channel image on your monitor. You can, for example, change the weighting of individual color channels in relation to one another.

8.5.2. Combining fluorescence images with a transmission image

1. Load one or more fluorescence images and the transmission image that you want to lay over the color channels.
2. Activate the transmission image in your software's document group.
3. Use the *Image > Combine Channels* command.
   - The *Combine Channels* dialog box opens.
   - If you want to use a true-color image as a transmission image, that image will be automatically selected in the *Transmission* list.

     When the transmission image is a gray-value image, it will be automatically entered in the *Available Images* table.
4. If necessary, choose the transmission image in the *Transmission* list.
5. Click once in the *Images* cell. You get a picklist containing all of the loaded images that you can combine with the selected transmission image. Select the fluorescence image you want.
6. If necessary, change the new fluorescence channel's properties. Give the channel a name and assign it a color.
7. Click the *OK* button, to create the resulting image.

   A new image document with the default name "Image_<serial No.>" is created.
   - In the document group, you can then see a superimposition of all of the images you've combined.
   - The resulting image is a multi-layer image with two image layers. Normally, the two image layers are not of the same image type. For this reason, the image has this icon  in the document group.



With the *Combine Channels* command, you combine several fluorescence images into a multi-channel image (**1**). If you've acquired the transmission image at the same place on the sample (**2**), you can combine it with the multi-channel image to make a multi-layer image (**3**).

Viewing a multi-layer image

- 

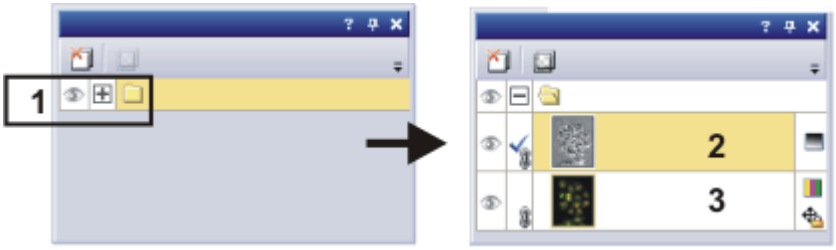
  The navigation bar is displayed at the top of the image window. You can find a button for showing and hiding the transmission image next to the button for the individual color channels. The eye icon indicates that the transmitted light image is currently visible.

8. Click this button in the navigation bar to hide the transmitted light image.
   - Now, you will only see the multi-channel fluorescence image.

9. Click this button and show the transmitted light image again.
   - Now, you see the transmitted light image superimposed on the multi-channel fluorescence image.

Moving the transmitted light image in relation to the multi-channel image

10. Use the *View > Tool Windows > Layers* command to make the *Layers* tool window appear.
11. In the *Layers* tool window, click the [+] sign (**1**) and open the image's layers.



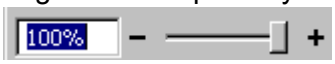
- You can now see the image's individual layers: Transmitted light image (**2**) and multi-channel image (**3**). The transmitted light image can't be seen, because it's absolutely transparent at the moment. The icon at the right side of the multi-channel image means that it is not possible to move the multi-channel image.

12. Select the transmitted light image in the *Layers* tool window.
13. Activate the *Toolbox* toolbar. To do this, you can use the *View > Toolbars > Toolbox* command.
14. Click the *Move Tool* button on the *Toolbox* toolbar.
    - On the image window, the mouse pointer will change its shape.
15. Move the whole image with the left mouse button depressed.
16. Click, for example, the *Selection Tool* button on the *Toolbox* toolbar to leave the move mode.

Changing the weighting between a transmission image and a multi-channel image

When you display the transmission image and the multi-channel image simultaneously in the image window, the transmission image will cover the multi-channel image, and for this reason, you can't see the multi-channel image. You can display both images transparently, and in that way be able to see parts of both images.

17. To start with, select the image layer in the *Layers* tool window. To do so, simply click the layer's name.
    - The layer you have selected will then be shown with a colored background in the tool window.
18. Then click the *Set Layer Opacity* button. You can find this button in the tool window's toolbar.
    - In the tool window a slide control will then appear, with which you can set the degree of transparency.



19. Use the slide control to set the degree of transparency you want. At a value of 100% the image layer is opaque. At a value of 0% the image layer will be completely faded out.
20. When you're satisfied with the transparency setting, click once on any place on the user interface.

Saving a multi-layer image

21. Use the *File > Save As* command to save your new multi-layer image. Always use the TIF or VSI file format when saving an image.

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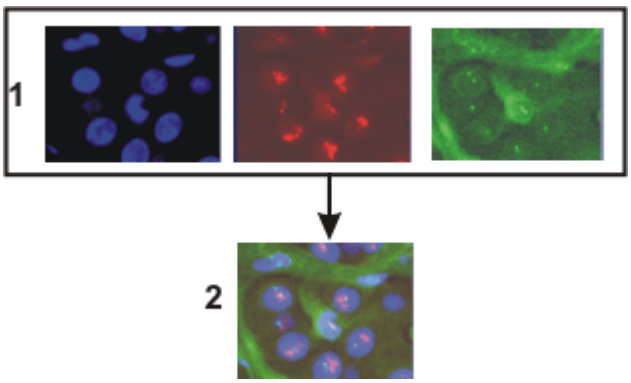
8.6. Acquiring multi-channel fluorescence images

Use the *Multi Channel* automatic acquisition process to acquire a multi-channel fluorescence image.

Example: Define a process for the acquisition of a multi-channel fluorescence image (with the Blue, Green, and Red color channels). When you set up the fluorescence sample, illuminate it as little as possible to minimize the bleaching effect.



At the top of the illustration you can see the individual fluorescence images (**1**).
Below, you can see the superimposition (**2**) of the separate fluorescence images.

Special hardware for acquiring multi-channel fluorescence images

Your software supports both image splitters and multi camera systems. Both systems enable you to acquire more than one color channel simultaneously, and to assemble them into a multi-channel fluorescence image.

Acquiring a multi-channel fluorescence image as an overview image

Prerequisite: You can only acquire an overview image of your sample if you are using a motorized XY stage.

When you are acquiring multi-channel fluorescence images, it can make sense to acquire an overview image of your sample as a multi-channel fluorescence image as well. This allows you to identify on the overview image the positions on the sample that are of interest to acquire as fluorescence image.

In the *Stage Navigator* tool window, click the *Acquire Multi Channel Overview* button to start acquiring the overview image. You define the acquisition process in the *Acquire Multi Channel Overview* dialog box.

8.6.1. Defining the acquisition process and acquiring multi-channel fluorescence images

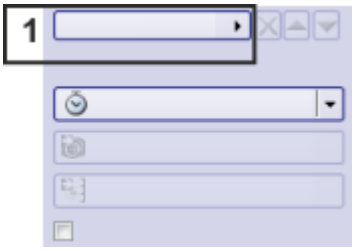
Selecting the acquisition process

1. Use the *View > Tool Windows > Process Manager* command to make the *Process Manager* tool window appear.
2. Select the *Automatic Processes* option.

3. Click the *Multi Channel* button.
   - The button will appear clicked. You can recognize this status by the button's colored background.
   - The [*C*] group will be automatically displayed in the tool window.
4. Should another acquisition process be active, e.g., *Z-Stack*, click the button to switch off the acquisition process.
   - The group with the various acquisition processes should, for example, now look like this:

Adding color channels

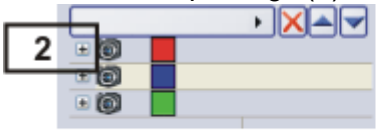
5. Click the *Add Channel* button (**1**).

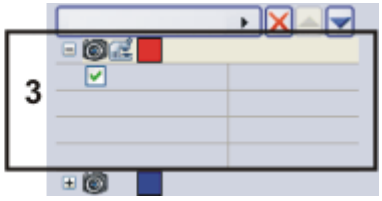


   - A context menu opens.
     The context menu lists all of the observation methods that are currently defined.
6. Select the color channel that is to be acquired first, e.g., Red.
7. Select the other channels (e.g., Green and Blue) in the same manner. Note that the fluorescence images are later on acquired in the same order as they are listed there.

Viewing the color channel settings

8. Click the small plus sign (**2**) next to the first color channel.



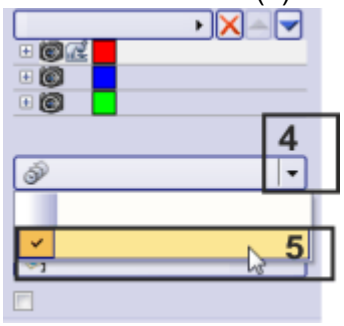


- The channel has now been activated (**3**). The active color channel will be shown highlighted in color in the tool window.
- The color channel entries in the *Process Manager* tool window are organized like a tree view. Expand an entry to open a list with additional information about the selected color channel.

- When you activate the color channel, you also automatically select the corresponding observation method. You can recognize which observation method is active, by the microscope icon . At the same time, this means that the microscope is now set up correctly for the acquisition of the fluorescence image for the first color channel.

Selecting the exposure times for the color channels

9. Click the small arrow next to the *Auto Exposure* button (**4**) and select the *On all channels* command (**5**) from the context menu.



- Your software now sets the observation method for each channel on the microscope and automatically determines the optimal exposure time.

- The icon on the *Auto Exposure* button now looks like this . You can click the button to redetermine the automatic exposure times for all of the channels after changing an objective, for example.
- The exposure times are adopted in the *Process Manager* tool window for each channel, and are applied when the channels are acquired.

10. If necessary: Correct the exposure time that was automatically determined for individual channels. To do so, click the *Exposure Time* field and enter the required exposure time.
    - The exposure times are saved separately for each channel. You can view these values for each channel later on, in the *Properties* tool window.

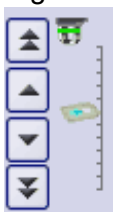
11. Finish the live mode. To do so, click the *Live* button in the *Camera Control* tool window.
    - The reflected light shutter will be closed.

Focusing a fluorescence sample

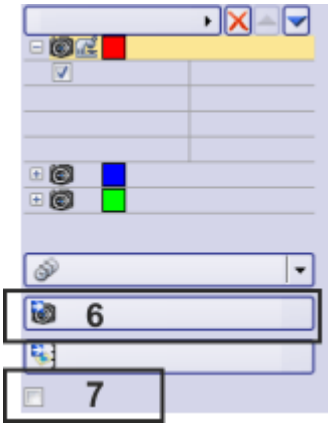
Prerequisite: You have a stage with a motorized Z-drive. If not, finish the process definition now.

12. Activate the first channel.
13. Switch to the live mode.
14. Bring the image into focus.

- If your microscope stage is equipped with a motorized Z-drive, a focus regulator will be at your disposal in the *Microscope Control* tool window.



15. Click the *Read Z-offset* (**6**) button in the *Process Manager* tool window to adopt the current Z-position of your microscope stage.
    - The current Z-position is adopted in the *Z-offset reference* field below the first channel.
    - Later, your software automatically moves to the Z-position specified in the *Z-offset reference* field before acquiring the image.
16. Select the *Use Z-offset* check box (**7**).



17. Activate the other channels, focus the sample and transfer the current Z-position of the microscope stage to the acquisition process.
    - The first color channel is always used as a reference for the Z-offset. Below the other color channels you can find the *Z-offset* value. It shows how the focus positions of the individual color channels differ from each other.
18. Finish the live mode.
    - The reflected light shutter will be closed.

Finishing the process definition

19. Click the *Save Process Definition* button in the toolbar at the top of the *Process Manager* tool window to save the acquisition parameters for the process that has just been defined.
    - A channel's definition contains an observation method, an exposure time, a gain value, and where necessary, a focus position. All of these settings will be saved together with the process definition.
    - You can now reuse the acquisition parameters for this acquisition process at any time, as long as the observation methods being used are available.
    - If one of the observation methods being used is no longer available, the

corresponding color channel is automatically switched off when you load a process definition.

Acquiring a multi-channel fluorescence image

Switching to a dark user interface

1. If you are disturbed by the light of your monitor, you can switch your software to a dark user interface. To do this, select the *View > Dark Application Skin* command and restart your software.

Defining the acquisition process

2. Define a process for the acquisition of a multi-channel fluorescence image, or load acquisition parameters that have already been saved. To do this, click the *Load Process Definition* button, located in the *Process Manager* tool window's toolbar.

Starting the acquisition process

3. In the *Process Manager* tool window, click the *Start* button.
   - If you use a non-motorized microscope, you'll receive several different messages about switching the mirror cube and opening and closing the shutter.
4. For microscopes that aren't motorized: Follow the instructions and make the necessary settings on your microscope.
   - The acquisition of the multi-channel fluorescence image starts immediately. The order of the color channels in the *Process Manager* tool window corresponds to the order in which the color channels were acquired.
   - The acquisition has been completed when you can again see the *Start* button in the *Process Manager* tool window.
   - The multi-channel fluorescence image will be automatically saved. You can set the storage directory in the *Acquisition Settings > Saving > Process Manager* dialog box. The preset file format is VSI.
   - The multi-channel fluorescence images that were acquired are displayed in the image window.

8.6.2. Acquiring a multi-channel fluorescence image together with a transmitted light image

Task: Acquire a transmitted light image, e.g., a phase contrast image, simultaneously with the multi-channel fluorescence image.

Defining the acquisition process

1. Define an acquisition process for a multi-channel fluorescence image. To do this, follow the step-by-step instructions described above.
   - In the *Process Manager* tool window, all of the defined fluorescence channels will be displayed in the [*C*] group.

Adding the acquisition of a transmitted light image to the acquisition process

2. Click the *Add Channel* button. Choose an observation method for the acquisition of a transmitted light image, e.g., phase contrast, differential interference contrast (DIC), or brightfield.

   - The transmitted light image is identified by this button to the right of the channel name.

   - The transmitted light channel is automatically added after the last fluorescence channel. You can also acquire the transmitted light channel before the fluorescence channels. To do this, use the button to the right of the *Add Channel* button to move the transmitted light image to the first position in the tool window.
3. Click on the transmitted light channel in the *Process Manager* tool window.
   - The channel has now been activated. Your microscope will be set in the transmitted light mode.
4. Click the small plus sign next to the transmitted light channel.
   - You'll now see a table with additional information about the transmitted light channel.
5. Make sure that the *Transmission overlay* check box has been selected. Only then is the transmitted light image assigned its own layer that lies over the fluorescence channels.

Note: If the transmitted light image is being acquired with a color camera, the *Transmission Overlay* check box must be selected. Otherwise the multi-channel fluorescence image can't be acquired.

Defining a transmitted light acquisition

6. Switch to the live mode.
7. Select manual exposure time in the *Camera Control* tool window. In order to set the sensitivity to the lowest ISO value, set the gain to the value of 0. Optimize the exposure time.

8. In the *Process Manager* tool window, click the *Read settings* button.
   - The exposure time will be adopted for the channel.

9. Finish the live mode.

Starting the acquisition process

10. In the *Process Manager* tool window, click the *Start* button.
    - Then, together with your fluorescence images, a transmitted light image will also be acquired and saved together with the multi-channel fluorescence image. The result of this acquisition process is a multi-layer image that you can view with the *Layers* tool window.

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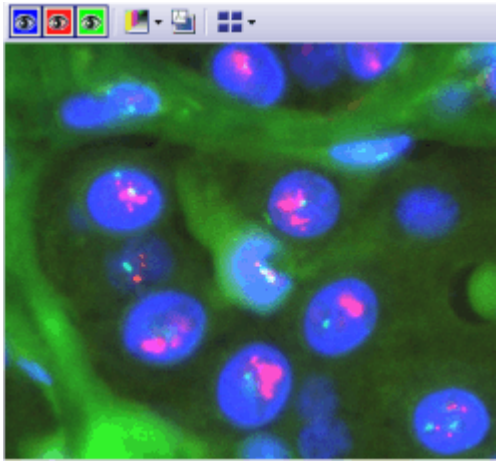
8.7. Working with multi-channel fluorescence images

Your software supports complex image types, for example multi-channel fluorescence images or multi-channel time stacks.

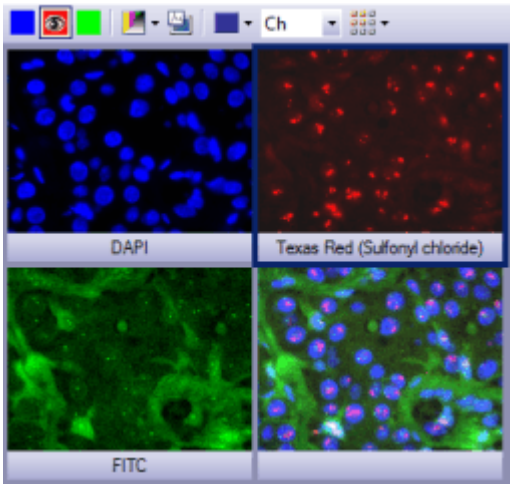
8.7.1. Viewing multi-channel fluorescence images

1. Load or acquire a multi-channel fluorescence image.
   - You will find step-by-step instructions on how to acquire multi-channel fluorescence images here.
   - All of the fluorescence images are superimposed on each other in the image window.



   - 

     The navigation bar is displayed at the top of the image window. It contains a button for each channel to enable you to display or hide that channel. The eye icon indicates that the channel is currently visible.
2. Click the color channel button in the navigation bar to have a color channel displayed or hidden. Take a look at all of the color channels one by one.
3. When you've finished, superimpose all of the channels again.
4. Click the *Tile View* button located in the navigation bar to change the image window view.
   - In the image window, you now see all of the color channels that have been acquired.



- In tile view, the buttons no longer affect the individual color channels. All of the color channels are always displayed.
- You can set whether the merged channels image is also displayed. Open the *Tools > Options > Images > View* dialog box. Clear the *Show merged channels* check box to hide the merged channels image.

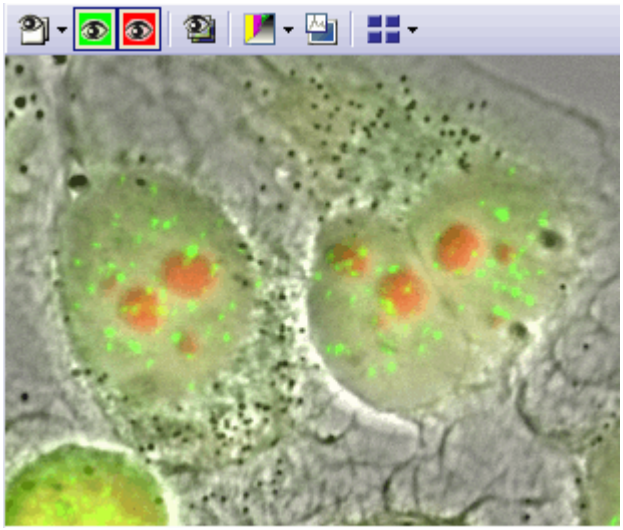
5. Compare the color channels.
6. Click the *Single Frame View* button on the navigation bar.
   - You will then once more see the superimposition of all of the color channels in the image window.

Viewing information on the individual color channels

7. Use the *View > Tool Windows > Properties* command to make the *Properties* tool window appear.
   - In the *Properties* tool window, you can find that every color channel has its own *Channel* information group.
8. If an information group is not displayed: Click the plus sign ⊞ to have all of the information displayed again.
   - The color channel's name, the corresponding wavelength, the observation method, and the exposure time can all be shown for each color channel.

8.7.2. Viewing a fluorescence image with a transmitted light image

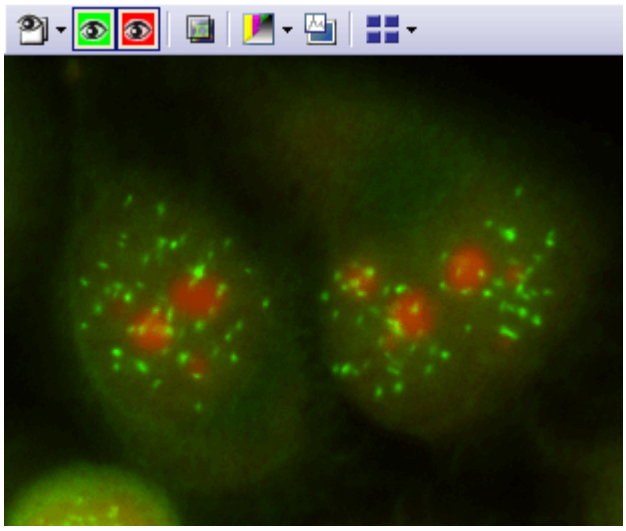
1. Load or acquire a fluorescence image with a transmitted light image.
2. Take a look at the multi-channel fluorescence image with the superimposed transmitted light image in the image window.



- 

  The navigation bar is displayed at the top of the image window. You can find a button for showing and hiding the transmission image **next to** the button for the individual color channels. You can find a button for showing and hiding image layers to the **left** of the buttons for the individual color channels. You can use this button to hide the multi-channel fluorescence image so that you can view the transmitted light image on its own.

3. Click this button in the navigation bar to hide the transmitted light image. The eye icon indicates that the transmitted light image is currently visible.
   - Now, you will only see the multi-channel fluorescence image.

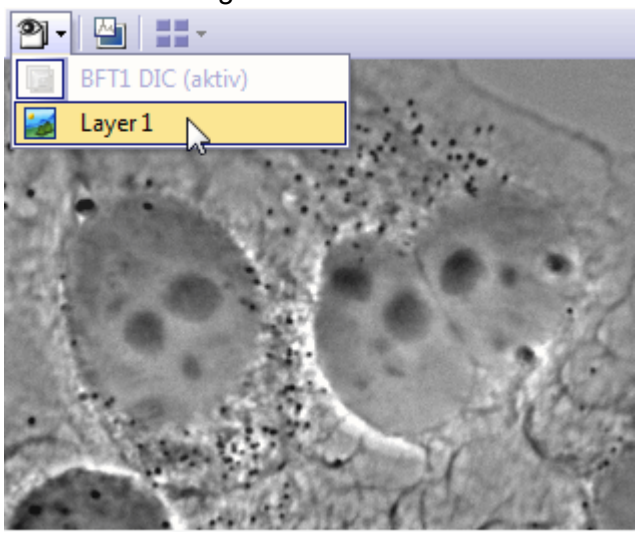


4. Click this button and show the transmitted light image again.

5. Click the *Set layer visibility* button in the navigation bar to open a list of all of the layers in the active image.
   - The image contains 2 image layers, the transmitted light image and the multi-channel fluorescence image. The multi-channel fluorescence image comprises a single image layer, even if the image contains more than one color channel.

- There is a button in front of every layer. The buttons in front of the visible layers are active. You can recognize this status by the button's background color. In this example, both of the layers are visible.

6. Select the entry for the multi-channel fluorescence image from the list.
   - Now, you will see only the transmitted light image. The multi-channel fluorescence image is hidden.

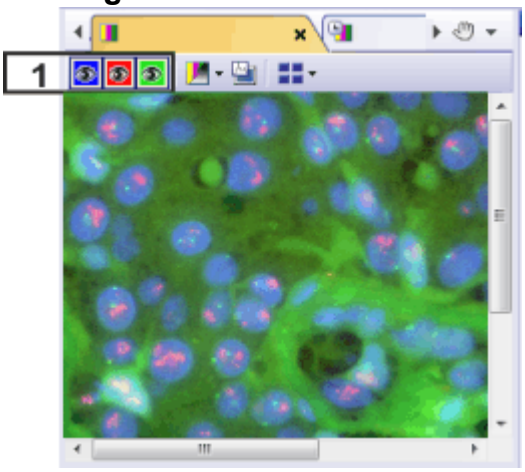


   - At least one image layer always has to be displayed. That's why the entry for the transmitted light image is grayed out. When the second image layer is hidden, the transmitted light image cannot be hidden as well.
7. Select the entry for the multi-channel fluorescence image from the list again to display both of the image layers again.
   - Alternatively, you can use the *Layers* tool window to show and hide image layers.

8.7.3. Setting the contrast for a multi-channel fluorescence image

Task: The multi-channel fluorescence image contains the 3 color channels DAPI, Texas Red and FITC. Take a look at each of the three channels individually, and next to each other. The green channel is too bright in comparison to the other color channels. Change the image contrast so that all of the color channels are optimally displayed.

Viewing individual channels



The navigation bar is displayed at the top of the image window. It contains a button for each channel in the image, to enable you to show or hide that channel. The eye icon indicates whether the channel is currently visible or not.

1. Click the color button on the navigation bar to show and hide a channel. Take a look at all of the three channels individually.

Optimizing the contrast in all of the channels simultaneously

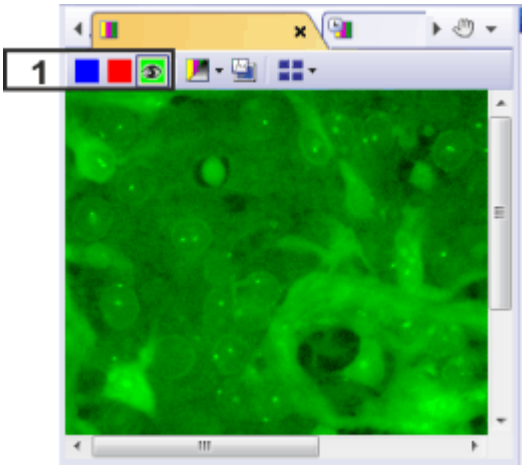
2. Click the *Adjust Display* button on the navigation bar in the image window.
   - This opens a small dialog box.

3. Click the *Auto Contrast* button.
   - A value of 0.1% in the *Right* field for example, means that in every channel 0.1% of the brightest pixels have been assigned at the maximum intensity, so that for the rest of the pixels the contrast will be increased.
   - You can set useful default values for the display of fluorescence images. To do this, select the *Tools > Options* command and select the *Images > Default Display Settings* entry in the tree view. In the *Display parameters* group, you can enter 10 in the *Left* field and 0.01 in the *Right* field, for example.
4. Change the value in the *Right* field step by step, and click the *Apply* button each time you do that. For fluorescence images, a smaller value of 0.01 in the *Right* field is generally a good choice, since then the few very bright pixels in each channel that contain the structure information won't be over-saturated.
5. Increase the value in the *Left* field step by step, and click the *Apply* button. For fluorescence images you can generally enter a high value in the *Left* field, since by doing so, you'll decrease the background fluctuations. A suitable value could be 10.

Optimizing the green channel

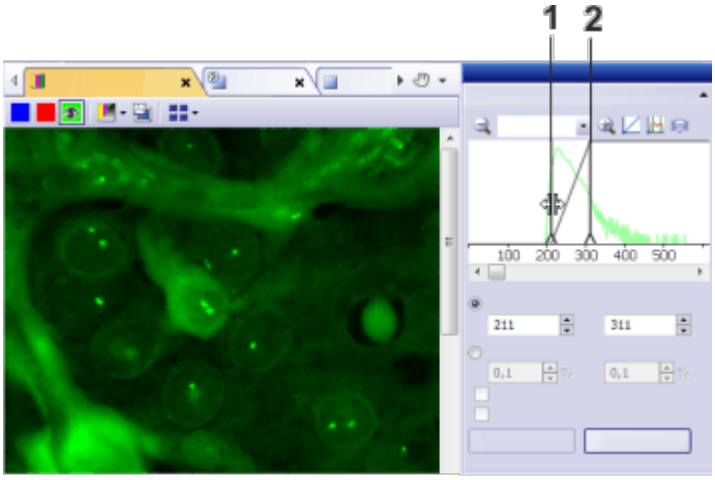
With multi-channel fluorescence images, you can individually set the way that each color channel appears in the image window. This enables you to accentuate a color channel in the merged channels image without changing the image data.

1. Display only the green channel in the image window. This means that you will now only be changing the appearance of the green channel.



Only the green channel has an eye icon (**1**). So now only the green channel is being displayed. This means that the contrast will only be changed for the green channel.

2. It's often easier to adjust the display manually with the help of the histogram. Use the *Image > Adjust Display* command to display the *Adjust Display* tool window.
   - The histogram of the green color channel is displayed at the top of the *Adjust Display* tool window. In the histogram, the number of pixels is plotted against the intensity. Therefore, it shows how many pixels are present in the image, and their intensity.
3. In the *Adjust Display* tool window, select the *Fixed Scaling* option.
4. You can alter the minimum and maximum values for the fixed scaling directly in the histogram. To do this, move the mouse pointer over one of the two vertical lines. Once the mouse cursor changes its shape - into a 'double-arrow'  , keeping the left mouse button depressed, you can move the line to where you want it. Note how the appearance of the image in the image window changes. Set the optimal appearance for the green channel.
5. Show all of the color channels so that you can see the merged channels image.



You can adjust the display in the histogram itself. To do this, move the lines (**1**) and (**2**). The values in the *Left* and *Right* fields are updated automatically.

Note: You do not change the actual image data when you adjust the display. The image will only be displayed differently on your monitor. The settings for the display will be saved along with the image, provided you save the image in the TIF or VSI format.

8.7.4. Creating and saving animations

When working with multi-dimensional images you can choose between different image window views. The tile view shows you the frames in a multi-dimensional image. Use the *Save Animation As* command to save the tile view as a movie in the AVI file format. You can find this command in the tile view's context menu.

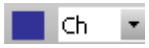
Task: Save a multi-channel Z-stack image as a movie. You want the color channels to be displayed side by side so that it's easy to compare the development of the fluorescence in the two color channels.

1. Load the multi-channel multi-dimensional image.
   - Multi-dimensional images automatically have their own navigation bar directly in the image window.
2. Open the *Tools > Options > Images > View* dialog box.

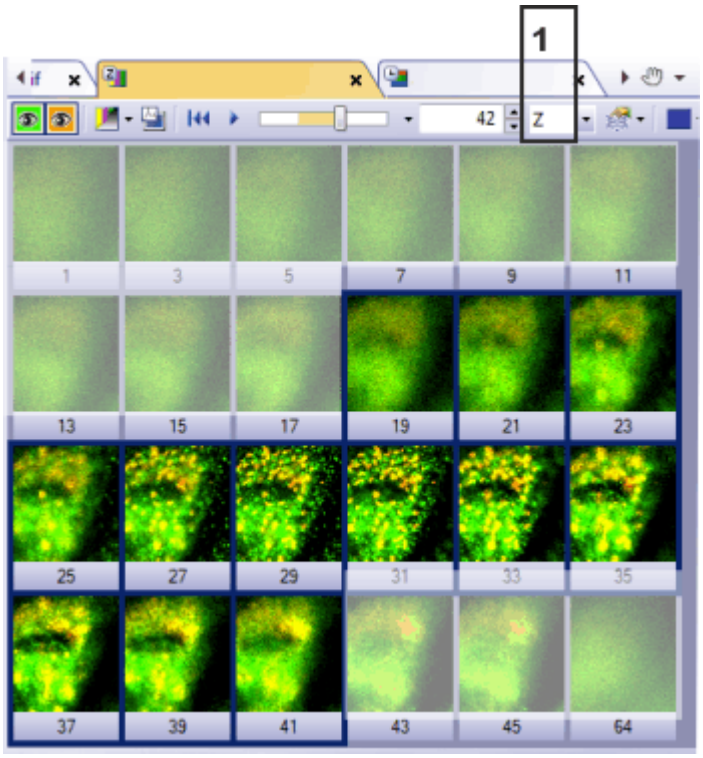
   Clear the *Show merged channels* check box. If you want to show a scale bar in the movie, select the *Display overlay elements of image view* check box.

   Close the dialog box with *OK*.
   - Now the tile view hides the merged channels image of the fluorescence channels. Now only the individual color channels are shown next to each other.
3. Check that the scale bar is shown. If necessary, use the *View > Scale Bar* command to show it in the image window.
4. In the image window's navigation bar click the small arrow next to the last button on this navigation bar to open a menu with commands you can use with image window views.

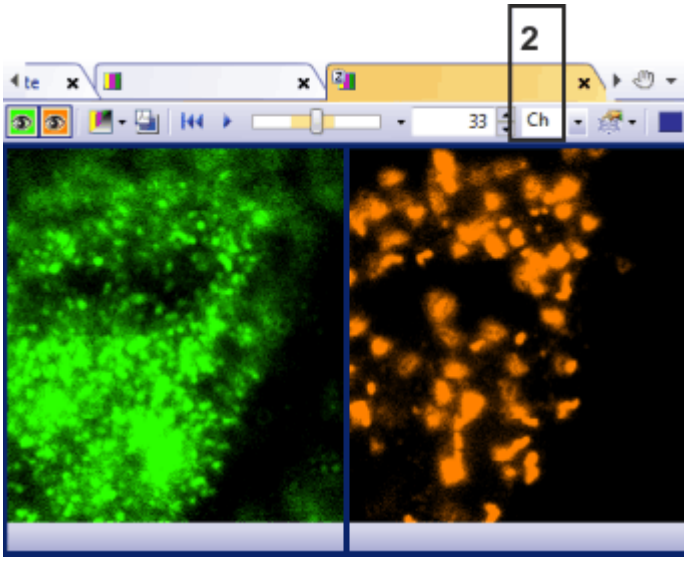
5. In this menu, select the *Tile View* command to switch to the tile view.



   - As soon as you've switched to the *Tile View* image window view, a picklist will appear in the navigation bar, next to the button with which you toggle the image window view. If the *Single Frame View* button is currently displayed, the picklist will look like this .
6. You can limit the number of frames that are saved in the movie. From the picklist, you can choose the image dimension that you want to display in tile view. Select the [Z] entry to display the frames of the Z-stack.
   - In the tile view, you can see the frames that belong to the different Z-positions. The merged channels image of the different color channels is displayed for each Z-position.
7. In the tile view, select the Z-frames that you want to save in the movie.
8. Select the [*Ch*] image dimension to display the color channels side by side. It is this image that will be saved as a movie.



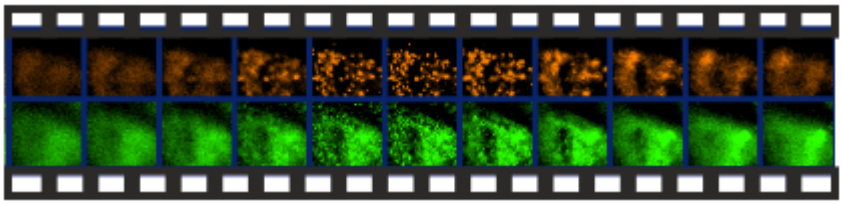
If you select the [*Z*] (**1**) image dimension for a multi-channel Z-stack image in the tile view, the color channels will be superimposed on each other and the individual Z-positions will be displayed. In this view, select the Z-positions that you want to save in the movie. In the example shown, image 19-41 have been selected.



Change the image dimension that is displayed in the tile view to create the movie. Select the image dimension [Ch] (**2**). The color channels are now displayed side by side. In this example, the color channels at Z-position 33 are being displayed.

9. Click the right mouse button and select the *Save Animation As* command in the context menu.
   - A dialog box for saving files opens.

10. The AVI video will, by default, be automatically compressed when it is saved. Click the *Options* button to open the *Select AVI Save Options* dialog box. You can view and change the AVI settings here.
    - Note: You can define the playback speed of the movie in the *Select AVI Save Options* dialog box. Enter the required playback speed in the *Frame rate selection* field.
11. In the *Select AVI Save Options* dialog box, define the required compression method, the frame rate, and the target image dimensions for the movie. Close the dialog box with *OK*.
12. Enter the name under which you want to save the move, and select the directory you want to save it to.
    - While the AVI video is being computed, you'll see a preview of the video. This can, where larger AVI videos are concerned, take some time. Watch the progress bar located in the preview.
    - When the movie has been rendered and saved, it will open in your software. You can play the movie in your software or in a different application program.



In the finished movie, both of the color channels are displayed side by side for different Z-positions. When you play the movie, you can see how the fluorescences are distributed in the sample.

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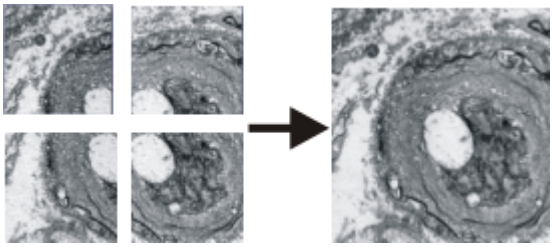
9. Creating stitched images

What is a stitched image?

If you acquire a stitched image, move the stage in a way that different, adjoining parts of the sample are shown. All of the images that are acquired are combined, just like a puzzle, into a stitched image. The stitched image will display a large sample segment in a higher XY-resolution than would be possible with a single acquisition.



The illustration shows left, four separate images. On the right you see the stitched image made up from the four images.

Creating a stitched image

Your software offers you several ways of creating a stitched image.

Acquiring a stitched image by moving the stage (Instant MIA)
Acquiring a stitched image without a motorized XY-stage (Manual MIA)
Acquiring a stitched image with a motorized XY-stage (XY-Positions/MIA)
Acquiring a stitched image with extended depth of focus
Automatically acquiring several stitched images
Combining individual images into a stitched image

Note: If image defects on the edge of an image decrease the quality of the stitched image or hinder the assembly of the individual images, you can clip these images during acquisition with *Subarray* mode in the *Camera Control* tool window.

Acquiring stitched images with the experiment manager

If your version of the software contains the *Experiment Manager* tool window, you can also use the experiment manager to acquire a stitched image.

9.1. Acquiring a stitched image by moving the stage (Instant MIA)

Preconditions

For the acquisition of stitched images, it's very important that your system has been correctly set up. If, for example, the shading correction wasn't performed correctly, the individual images create a tiled effect in the stitched image. It's also very important that the camera is aligned parallel to the stage's XY-axes. When the camera is askew in relation to the stage, the individual images in the stitched image will also be askew in relation to one another. The angle between camera and stage should be smaller than 1°.

Making settings for the acquisition of an image

1. Switch to the *Acquisition* layout. To do this, use the *View > Layout > Acquisition* command, for example. The *Camera Control* tool window and the *Process Manager* tool window are displayed automatically.
2. Use the default acquisition settings for the *Instant MIA* process. To do so, open the *Acquisition Settings > Acquisition > Instant MIA* dialog box. Click the *Default* button and close the dialog box.

   - You can open this dialog box, for example, via the *Process Manager* tool window. In the tool window's toolbar, click the *Acquisition Settings* button. Select the *Acquisition > Instant MIA* entry in the tree view.
3. Select the microscope settings you want. In particular, select the required magnification. If you have defined observation methods, select the required observation method.
   - In this case, the background color of the stitched image depends on the observation method that has been selected. The background is automatically black for all fluorescence observation methods and all darkfield observation methods. The background is white for all other observation methods.

Selecting, configuring and starting the acquisition process

4. Activate the *Process Manager* tool window.

5. Select the *Manual Processes* option, and click the *Instant MIA* button.
6. Check the configuration of this acquisition process.

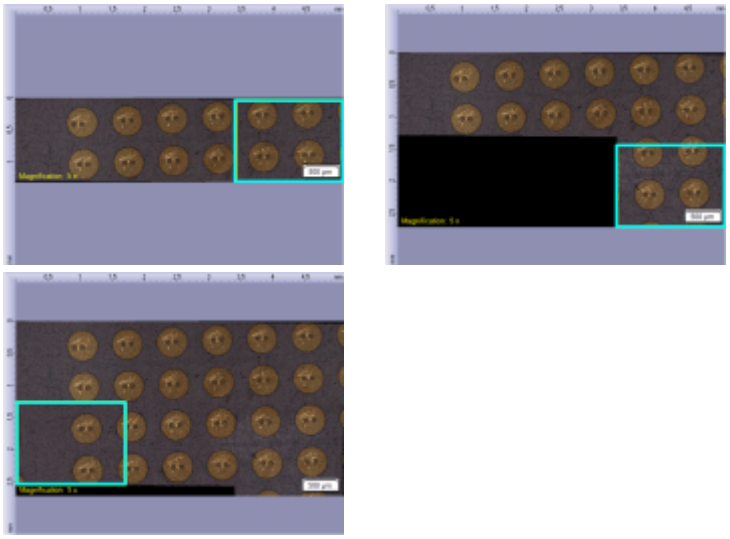
7. Click the *Start* button.
   - The *Adjust Acquisition Conditions* dialog box opens.
   - Your software will automatically switch to the live mode.
   - The camera resolution is set to the value that is specified in the acquisition settings.
   - You can't use HDR with the *Instant MIA* acquisition process. If HDR is activated when you start this acquisition process, you receive an error message to this effect. Deactivate HDR in the *Camera Control* tool window and restart the acquisition process.
   - Your software checks how much storage capacity is available. If too little storage capacity is available, an error message appears.
8. Select the optimal settings for your acquisition in the *Camera Control* tool window. You can still adjust the camera resolution as well.
   - The settings are applied to all of the individual images that make up the stitched image (exposure time, resolution, subarray, the white balance).
   - The focus setting that is now made is, by default, also used for all of the individual images that make up the stitched image. The autofocus function is deactivated during the *Instant MIA* acquisition process. You can, however, still adjust the focus manually while the acquisition process is running. This is only possible in a special focus view.

Note: It's especially important that the sample is well exposed and that the current exposure time is as short as possible. If the exposure time is too long, you receive an error message.

9. Find the position on the sample at which you want to start acquiring the stitched image.
10. In the *Adjust Acquisition Conditions* dialog box, click the *Start* button.
    - The first image of the stitched image is displayed in the image window.
    - Most of the software's functions are now not available. Camera control is also locked.
    - The software switches to a special MIA image view. This view uses the MIA cursor. It consists of a square frame that can have different colors (see the table below).

Acquiring a stitched image

11. Slowly move the stage to the next position on the sample.
    - The camera acquires images continuously as long as you move the stage. The individual images are immediately assembled. You can watch how the stitched image grows, in the image window.
    - If required, use the mouse wheel to zoom in to or out of the stitched image. Alternatively, you can also use the *Zoom* toolbar for this.



Display of the stitched image during the *Instant MIA* acquisition process. The MIA cursor indicates the status of the image acquisition.

12. Pay attention to the MIA cursor. The color of the frame indicates the status of the image acquisition.

	A light blue frame means that there are no problems with assembling the stitched image.
	A yellow frame means that it's still possible to assemble the images. The settings, however, aren't optimal. It could be that the stage was moved too quickly, for example.

	An orange frame means that the stitching position was temporarily lost. It could be that the stage was moved too quickly, for example, or that the sample has too little image information at the current stage position for the images to be assembled. However, your software can often find the stitching position again in this state by its own means.
	A red frame indicates that the stitching position was definitely lost. Your software can't find the stitching position again in this state by its own means. However, in certain cases, you can manually bring your software into a state where the stitching position is found again. Alternatively you can now finish the *Instant MIA* acquisition process. The stitched image contains all information that had been acquired until the stitching position was lost.

Adjusting the focus on the sample

13. If you need to adjust the focus on the sample (for example, if you navigate to a slightly thicker position on the sample), click the *Focus View* button. You'll find the button in the *Instant MIA* group, located in the *Process Manager* tool window.
    - The *Focus View* button now becomes the *MIA Image View* button.
14. Adjust the focus on the image. Either use the focus knob on the microscope for this, or if your microscope has a motorized Z-drive, use the slide control in the *Microscope Control* tool window. The autofocus function can't be used while the *Instant MIA* acquisition process is activated.
    - In focus view, the live-image is displayed in a new tab. The MIA image view remains on its own tab in the image window. The stitched image, however, is not refreshed as long as you stay in focus view.
15. When you've adjusted the focus on the sample, click the *MIA Image View* button.
    - Switch back to the MIA image view and you can continue with the image acquisition.

Note: The *Instant MIA* acquisition process can’t run indefinitely. The acquisition process ends automatically after about 30 minutes.

Stopping image acquisition

16. Click the *Stop* button when you want to end the acquisition of the stitched image.
    - You see the completed stitched image, in the image window. It is usually not rectangular, but instead contains empty areas on its borders. In the stitched image, these areas are by default colored in white, or in black with dark field images.

      You can also select any background color you want. To do so, select the *Select background color* check box in the acquisition settings.
    - The stitched image will, by default, be automatically saved. The storage directory is shown in the *Acquisition Settings > Saving > Process Manager* dialog box. The preset file format is VSI.
    - The images composing the stitched image will not be saved separately.

9.2. Acquiring a stitched image without a motorized XY-stage (Manual MIA)

Task: You want to acquire an image of a large sample area. Use the *Manual MIA* acquisition process to acquire several individual images of adjoining positions on the sample, and to have them combined into a stitched image. MIA stands for Multiple Image Alignment.

Prerequisite

The camera is aligned parallel to the XY-stage. The angle between camera and stage should be smaller than 1°.

1. Switch to the *Acquisition* layout. To do this, use the *View > Layout > Acquisition* command, for example.

Selecting microscope settings

2. Select the microscope settings you want. In particular, select the required magnification. To do this, on the *Microscope Control* toolbar, click the button with the objective that you want to use for the acquisition of the stitched image. If you are using a magnification changer, you will also have to select the magnification value used.
   If you have defined observation methods, select the required observation method instead.
   - In this case, the background color of the stitched image depends on the observation method that has been selected. The background is automatically black for all fluorescence observation methods and all darkfield observation methods. The background is white for all other observation methods.

Setting the image quality

3. Switch to the live mode, and select the optimal settings for your acquisition, in the *Camera Control* tool window. Pay special attention to setting the correct exposure time. This exposure time will be used for all of the stitched image's individual images.
4. Find the position on the sample at which you want to start acquiring the stitched image.
5. Finish the live mode.

Selecting the acquisition process

6. Activate the *Process Manager* tool window.
7. Select the *Manual Processes* option.
8.  Click the *Manual MIA* button.
   - The button will appear clicked. You can recognize this status by the button's colored background.
   - The *Manual MIA* group will be automatically displayed in the tool window.
   - Should the *Instant EFI* acquisition process have been active, it will be automatically switched off. You can, however, use images with extended depth

of focus for the stitched image. To do this, before you acquire each of the individual images, click the *Instant EFI* button located in the *Manual MIA* group.

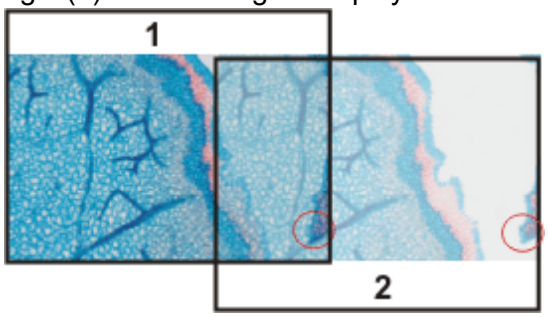
Setting the acquisition parameters

9. Make quite certain that the *Auto Align* button appears clicked. It should then look like this:
   - Then your software will search for the same image structures in neighboring individual images. The stitched image will be put together in such a way that image areas that are the same will be superimposed.

Acquiring a stitched image

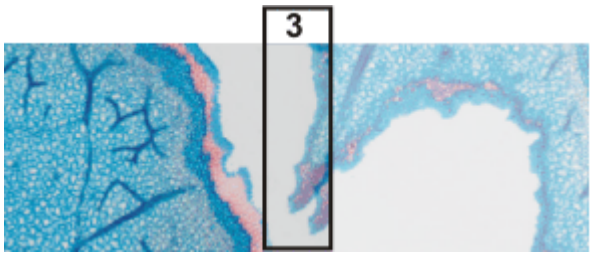
10. Click the *Start* button.
    - Your software switches into the live mode.
11. Bring the sample into focus.

12. Click on one of the arrow buttons to set the side of the current image at which the next image is to be arranged. For example, click this button if the next image is to be laid to the right of the current image.
    - Your system now acquires an image at the current position on the sample. In the image window you now see on the left (**1**) the acquired image, and on the right (**2**) the live-image is displayed.



Because you haven't moved the sample, the live-image is still displaying the current position on the sample. This means that you are now looking at two displays of the current image.
The two images overlap. Because the live-image is transparent, both images are displayed in the overlap area.

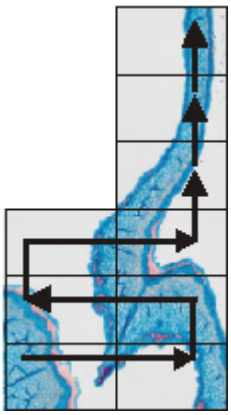
13. Make a note of a significant structure on the live-image's right border. You will find the same sample structure in the overlap area. On the illustration, a significant structure has been indicated by a circle.
14. Now move the stage very slowly to make the structure on the live-image move to the left. Keep moving the stage until the image structures in the overlap area lie as exactly over each other as possible. The image structures need not lie precisely over each other, since your software will match the individual images with each other.
    - In the overlap area (**3**), the same image segments are shown now. This enables your software to seamlessly combine the two images.



- You can reverse the direction in which your stage moves, in the *Device Settings > Stage* dialog box. Depending on how you can best orient yourself, the live-image will then move to the left or to the right, when you move your stage to the right.

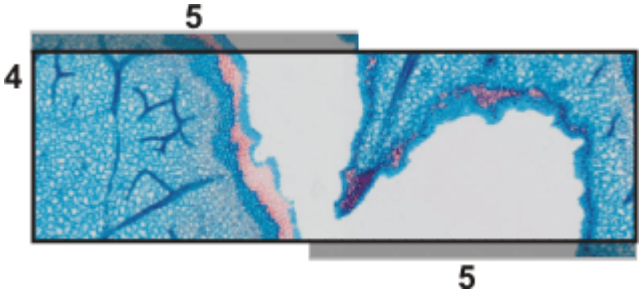
15. Check whether both images have been correctly combined. Otherwise, you can undo the last step by using the *Undo last frame* button. You can then move the stage again, and match the structures better.
    - During the acquisition, you can change the current stitched image's zoom factor, e.g., to see certain parts in the overlap area better.
16. Define your way through the sample, with the arrow buttons, and follow that with the stage.

    In this manner, you can display a sample in any form you like in the stitched image. The illustration shows a stitched image that is made up of 9 individual images, and the stage path.



17. Click the *Stop* button when you want to end the acquisition of the stitched image.
    - You see the completed stitched image (**4**) in the image window.

      Since the individual images can lie a little askew of each other, the stitched image isn't as a rule, rectangular, but contains empty areas on its borders (**5**). These areas will, as a rule, be cut off in the stitched image.

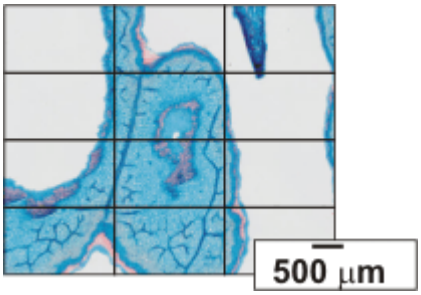


- The stitched image will, by default, be automatically saved. The storage directory is shown in the *Acquisition Settings > Saving > Process Manager* dialog box. The preset file format is VSI.

Properties of the stitched image

- By default, in the overlap area, the intensity values of two adjoining individual images will be matched with each other to make the image's overall impression homogeneous.
- Stitched images are calibrated. This means that you can measure distances and objects on a stitched image.

9.3. Acquiring a stitched image with a motorized XY-stage (XY-Positions/MIA)



Task: You want to acquire an image of a large sample area. Use the automatic *XY-Positions/MIA* acquisition process to scan an area of the sample and to have adjoining images combined into one stitched image. MIA stands for Multiple Image Alignment.

Prerequisite: You can only use the *XY-Positions/MIA* acquisition process if your microscope is equipped with a motorized XY-stage.

Preconditions

- The stage has been set up and initialized, i.e. its stage limits have been defined.
- The camera is aligned parallel to the XY-stage. The angle between camera and stage should be smaller than 1°.
- The shading correction has been set up.

1. Switch to the *Acquisition* layout. To do this, use the *View > Layout > Acquisition* command, for example.

Selecting microscope settings

2. Select the microscope settings you want. In particular, select the required magnification. To do this, on the *Microscope Control* toolbar, click the button with the objective that you want to use for the acquisition of the stitched image. If you are using a magnification changer, you will also have to select the magnification value used.
   If you have defined observation methods, select the required observation method instead.

- In this case, the background color of the stitched image depends on the observation method that has been selected. The background is automatically black for all fluorescence observation methods and all darkfield observation methods. By default, the background is white for all other observation methods.

Selecting the acquisition process

3. Activate the *Process Manager* tool window.
4. Select the *Automatic Processes* option.

5. Click the *XY-Positions/MIA* button.
   - The button will appear clicked. You can recognize this status by the button's colored background.
   - The *XY* group will be automatically displayed in the tool window.

Using the software autofocus

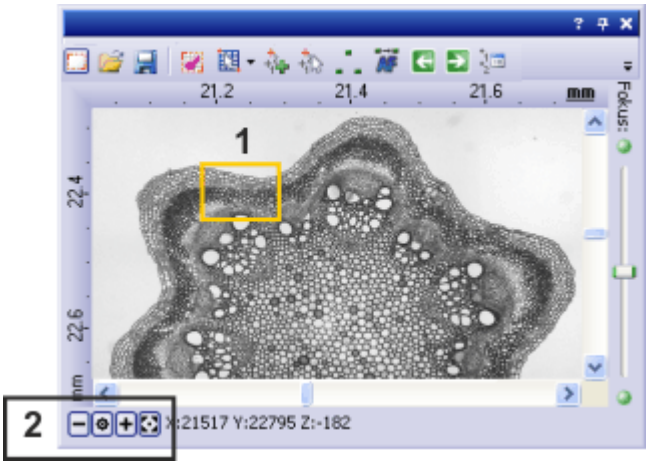
6. If your microscope is equipped with a motorized Z-drive, you can switch on a software autofocus.

   In the *Process Manager* tool window, click the *Autofocus* button.
   - The *Autofocus* group will be automatically displayed in the tool window.
7. In the *Autofocus* group, select the *Multiposition / MIA autofocus* check box.

   If the sample surface is not plane or if it is inclined to the objective, choose the *Every MIA frame* option. Now, the software autofocus will be performed before every image acquisition.

Putting the stage navigator on display

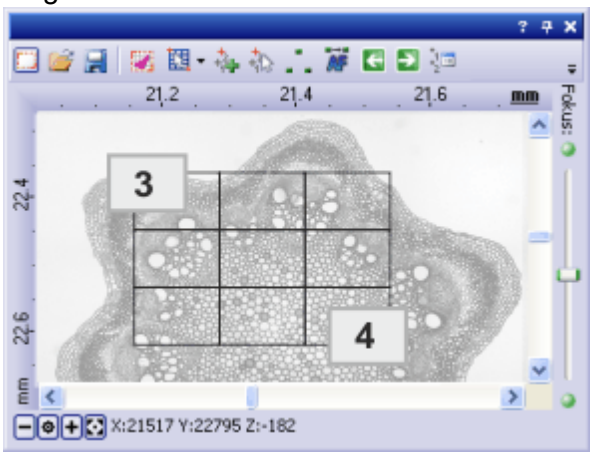
1. In the *Process Manager* tool window, click this button .
   - The *Stage Navigator* tool window is shown. When you have acquired an overview image of your sample, you will see this area of the image in the stage navigator's image segment.
2. Set the magnification for the image segment in the *Stage Navigator* tool window. To do this, use the zoom buttons at the bottom left of the tool window (**2**).



The current stage position is indicated by a yellow rectangle in the image segment (**1**). You should choose a magnification that enables you to see this rectangle clearly.

Defining the MIA scan area

3. In the *Process Manager* tool window, click this button .
   - The system will automatically switch into the live mode.
   - The *Define MIA Scanning Area* dialog box opens.
4. Move the XY-stage to the top left-hand corner of the MIA scan area you want (**3**).
5. Focus, then select the optimal settings for your acquisition in the *Camera Control* tool window. Pay special attention to setting the correct exposure time. This exposure time will be used for all of the stitched image's individual images.
6. Confirm the starting position in the *Define MIA Scanning Area* dialog box, with *OK*.
7. Move the XY-stage to the bottom right-hand corner of the MIA scan area (**4**). Confirm this position in the *Define MIA Scanning Area* dialog box, with *OK*.
   - In the *Stage Navigator* tool window, the MIA scan areas that have been defined are displayed. Here, you can immediately see how many individual images are required for the acquisition of the stitched image, when the current magnification is used.



Acquiring a stitched image

8. Click the *Start* button.
   - The acquisition begins immediately. The individual images are acquired, then immediately stitched. You can watch how the stitched image grows, in the image window.
   - In the status bar at the bottom left of the user interface, you can find a progress bar, the number of images already acquired, and the total number of frames (e.g., 3/9).

   - The acquisition has been completed when you can once more see the *Start* button in the *Process Manager* tool window, and the progress bar is no longer displayed.
   - You see the completed stitched image, in the image window. The individual images won't be saved separately.
   - The stitched image will, by default, be automatically saved. The storage directory is shown in the *Acquisition Settings > Saving > Process Manager* dialog box. The preset file format is VSI.

9.4. Acquiring a stitched image with extended depth of focus

When you acquire a stitched image of a thick section or an uneven surface, in some cases not all of the areas of the sample will be sharply displayed. In this case, you can combine the acquisition of a stitched image with the *EFI* (Extended Focal Imaging) acquisition process. By doing this, you'll make sure that the stitched image is sharply focused everywhere.

Note: The acquisition of a stitched image with extended depth of focus, is both with and without, a motorized XY-stage, possible.

Without a motorized XY-stage

1. Start the *Manual MIA* acquisition process.
2. Click the *Instant EFI* button, in the *Manual MIA* group.
   - The *Instant EFI* acquisition process will start at once. Instead of the live-image, you now see the EFI image.
3. Now move your microscope's Z-drive slowly and change the focusing of the image. Observe how the EFI image builds itself up.
   - For each image that is acquired, the sharpest image segment is adopted in the EFI image.
4. When all of the image structures are sharply displayed, click one of the direction arrows in the *Manual MIA* group to continue with the acquisition of the stitched image.

Note: You now see the live-image with the last focus settings. That means that normally, the live-image won't be in focus.

5. Bring the image into focus.
6. Repeat the last steps for each of the stitched image's individual images for which you want to use the *Instant EFI* acquisition process.

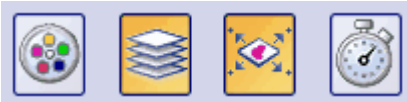
7. Click the *Stop* button when you want to end the acquisition of the stitched image.
   - You see the completed stitched image, in the image window.

With a motorized XY-stage

Prerequisite: You can only use the *EFI* acquisition process when your stage is equipped with a motorized Z-drive.

1. Select the *XY-Positions/MIA* acquisition process.
2. Define an MIA scan area.
3. Additionally, select the *Z-Stack* acquisition process.
   - In the group with the different acquisition processes, two of them are now

active:



4. Define all of the parameters for the Z-stack's acquisition.
   - You can find a step-by-step instruction for doing this here.
5. In the [*Z*] group, select the *Extended Focal Imaging* check box.
6. Click the *Start* button to begin the acquisition of the stitched image.
   - At each of the MIA scan area's stage positions, a Z-stack will first be acquired, then the EFI image calculated from it. The EFI images will be combined into a stitched image.
   - When the acquisition process has been completed, you'll see the finished stitched image in the image window.

9.5. Automatically acquiring several stitched images

You can define several MIA scan areas on the sample. When the acquisition has started, all of the MIA scan areas will be moved to, one after the other, and a stitched image will be acquired at every position.

1. Select the *XY-Positions/MIA* acquisition process.
2. Define several MIA scan areas.

   Begin with the area of the sample that is to be scanned first.

Putting the stage navigator on display

3. In the *Process Manager* tool window, click this button .
   - The *Stage Navigator* tool window is shown. When you have acquired an overview image of your sample, you will see this area of the image in the stage navigator's image segment.
   - In the *Stage Navigator* tool window, the MIA scan areas that have been defined are displayed. They are numbered serially in the order in which they were defined.

Acquiring stitched images

4. Click the *Start* button to begin the acquisition of the stitched image.
   - Each of the MIA scan areas will now be scanned, and the stitched image created. The scan areas will be scanned in the order that is predefined by the numbering.
   - All of the stitched images will be acquired with the current camera, and current acquisition settings.
   - When the acquisition process has been completed, you'll find a stitched image for each of the MIA scan areas, in the document group.

9.6. Combining individual images into a stitched image

Use the *Process > Multiple Image Alignment* menu command to have several separate images combined, as with a puzzle, into a stitched image. The individual images will be combined in their full XY-resolution. The stitched image will thus display a large sample segment in a higher XY-resolution than would be possible with a single acquisition.

Acquiring images

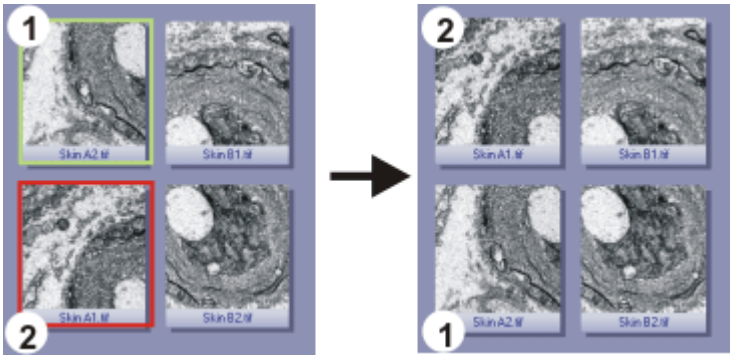
1. Load the images you want to combine or acquire a suitable set of images.
   - All of the images you want to combine must be of the same image type. You can't, e.g., have a gray-value image combined with a true-color image.
   - When you acquire the images, number their names sequentially, e.g., "Image001", "Image002" and so on. In many cases, the images will then already be arranged in the right order in the *Multiple Image Alignment* dialog box.

Selecting images

2. Open the *Gallery* tool window. To do this, you can use the *View > Tool Windows > Gallery* command.
3. Select all of the images you want to combine, in the *Gallery* tool window.

Assembling images

4. Use the *Process > Multiple Image Alignment* command. This command is only active when more than one image of the same image type has been selected.
   - The dialog box's stitching area will display a preview of the individual images.
5. If necessary, while keeping your left mouse button depressed, drag on the bottom left-hand corner of the dialog window to enlarge it. Alternatively, double click the header of the dialog box to enlarge the dialog box to full-screen size.
6. Check whether the images' positions are correct. You can change the arrangement of the individual images, e.g., by exchanging two images in the stitching area using Drag&Drop.



The illustration shows the stitching area with four individual images. On the left, the images 1 and 2 are not in the correct position. Image 1 (green frame) will therefore be dragged onto image 2 (red frame). On the right, you see the stitching area after the two images have been interchanged.

7. When the individual images overlap, select the *Correlation* entry in the *Output > Alignment* list.

   Then your software will search for the same image structures in neighboring individual images. The stitched image will be put together in such a way that image areas that are the same will be superimposed.
8. Click the *OK* button to carry out the automatic image alignment.
   - The *Multiple Image Alignment - Manual Align* dialog box opens.
   - The stitched image will be displayed.

Checking a stitched image

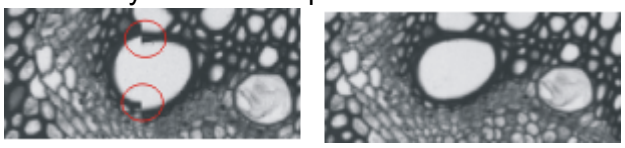
9. Check the stitched image on display.

   Use the zoom buttons in the dialog box to zoom in the stitched image in the dialog box.

10. Should individual images have been incorrectly assembled, you can manually shift one or more of them, in respect to one another.

    To do this, click in the image you want to shift, then drag it with your left mouse button depressed, in the required direction.
    - The currently selected image will be displayed semi-transparently to make it easier for you to find the point of contact with the neighboring image.

      
    - Two images were not correctly aligned with each other. There is a misalignment. When the manual alignment has been made, the two images fit together seamlessly.
11. Select the *Cut Edges* check box to clip the image in such a way that there are no longer any empty areas visible on its borders.
    - In the preview, the image edges that are to be clipped will be displayed semi-transparently.
12. Select the *Equalize* check box if the images aren't homogeneously illuminated. Then the intensity values of the individual images will be matched with one another, which will make the background appear more homogeneous.
13. Click the *OK* button.
    - A new image with the name *Image_<consecutive No.>* will be created.

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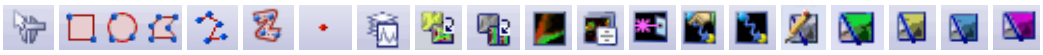
10. Life Science Applications

The *Life Science Application* toolbar offers you various evaluation methods for your images. If this toolbar is not displayed, use the *View > Toolbars > Live Science Applications* command.



The following table lists the buttons which are available by default on the toolbar.

	Select Measurement Objects	Click this button to select existing measurement objects and ROIs on an image. You can edit and delete selected measurement objects and ROIs or save them in a parameter set. The standard MS-Windows conventions for multiple selection apply for the selection of objects.
	New ROIs	Use one of several options to define an image segment in the active image as a region of interest (ROI).
		Please note that you can also define a ROI that measures a single point or a line.
	Intensity Profile	An intensity profile shows how the intensity within one, or within several image segments (ROIs), changes over a period of time or over the different Z-positions.
	Fluorescence Unmixing	Use fluorescence unmixing to remove spectral mixing from a multi-channel fluorescence image.
	Brightfield Unmixing	Use brightfield unmixing to break down a brightfield image containing three different colors into its individual color components.
	Colocalization	On a multi-channel fluorescence image, measure the colocalization to identify image segments where the individual fluorescences overlap.
	Ratio Analysis	Measure how the calcium ions concentration is changing in a time stack.
	FRAP analysis	Normalize the intensity profile from a FRAP experiment and analyze it. You can export the results in different output formats.
	FRET Correction FRET Analysis	Carry out a FRET analysis.
	Verify Deconvolution Channel	For the active image, check whether all of the parameters have been correctly defined which are necessary for successful deconvolution.

	Parameters	
	2D Deconvolution	Use a deconvolution filter to remove disturbing diffused light from an image.
	Nearest Neighbor	
	Wiener	
	Constrained Iterative Filter	

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10.1. Intensity profiles

10.1.1. Overview - Intensity profile

With the *Measure > Intensity Profile* command, you can measure the intensity profile over the time (time stack) or over the different Z-positions (Z-stack). An image series can be a time stack or a Z-stack.

What exactly is an intensity profile?

To calculate an intensity profile, all of the pixels within a specific image segment will be evaluated. Your software can determine the mean intensity of all of the pixels. Intensities with a value of 0 are interpreted as being part of the background and are ignored by the computation.
As a result you will obtain an intensity profile that shows how the intensity within one, or within several image segments, changes over a period of time or over the different Z-positions.

Before using the command

Before you can measure an intensity profile, you have to define this image segment. To do this, define one or more ROIs (Regions Of Interest) on the image. To define these ROIs, you can use the appropriate buttons on the *Life Science Applications* toolbar.

Supported image types

With the *Measure > Intensity Profile* command, you can measure the following image types:

- Time stacks, whose frames are gray-value images.
- Z-Stacks, whose frames are gray-value images.
- Multi-channel Z-stack
- Multi-channel time stacks

Prerequisite: The command is only available for monochrome images. If needed, use the *Image > Mode > Grayscale* command to convert an image into a gray-value image.

Usage examples

1. You can use intensity profiles to measure how concentrations change with time. For example, when you make experiments with triggering the calcium flow with ATR, and use suitable fluorescence stains.
2. If you purchased the *Photo Manipulation* solution along with your software, you can selectively bleach particular areas on your sample with a laser. A FRAP experiment of this nature produces a time stack. You can analyze the resulting time stack by calculating an intensity profile of the bleached areas on the sample. You can find more information on FRAP here.

Intensity profiles and datasets

You can compute many different intensity profiles on an image at the same time. For example, with a multi-channel time stack, you can compute an intensity profile for each image segment (ROI) and on each channel.

All of the intensity profiles that have been computed are assembled into one dataset. Each time that you click the *Execute* button in the *Intensity Profile* tool window, you compute one or more intensity profiles and thereby automatically create a new dataset each time. All of the datasets that have been computed appear in a list in the *Intensity Profile* tool window's toolbar.

The datasets remain available until you delete them or close your software. You can save datasets and the intensity profiles that they contain in a file that can be reloaded into the *Intensity Profile* tool window at a later point in time.

Intensity profiles and the experiment manager

Prerequisite: The *Experiment Manager* tool window is only available with the highest software package.

You can use your software to implement complex acquisition processes. Use the Experiment Manager to define and run complex experiments involving image acquisition with your software.

You can insert the *Intensity Profile* command into an experiment plan to, for example, acquire time stacks at different positions on the sample and then compute their intensity profiles.

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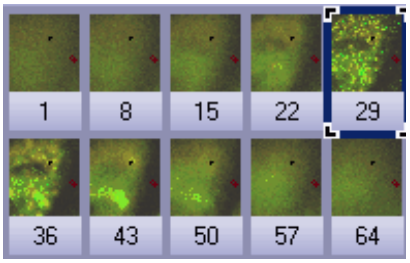
10.1.2. Measuring an intensity profile on a multi-channel Z-stack

Example: You have acquired a focus series for several fluorescences. You want to know how the intensity develops at a variety of positions on the sample at a variety of Z-positions.



The illustration shows an overview over the frames in a multi-channel Z-stack. The multi-channel image contains a red and a blue color channel. For the acquisition of the Z-stack a through-focus series was taken of the sample. The sample can only be seen clearly, and sharply focused, in the middle of the Z-stack.

The following process flow chart displays the basic steps of the process.

Preparing the analysis

Load the image that you want to measure.
Find a suitable frame on which to define the ROIs.

▼

Defining ROIs (Region Of Interest)

On your image, define the areas whose intensity profile is to be measured.

▼

Calculating and viewing an intensity profile

Define the settings for the calculation of the intensity profile.
Specify how the intensity profiles should be displayed in the *Intensity Profile* tool window.

▼

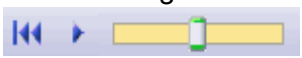
Exporting and saving intensity profiles

Preparing the analysis

1. Several example images were supplied together with your software. You can follow these step-by-step instructions using the PeroxysomOrganelles.tif example image. This example image is a multi-channel Z-stack image.
   - You can find more information on example images here.
   - When you load a multi-channel Z-stack, it will be automatically displayed in the Single Frame View in the image window.

Displaying a suitable image for the definition of the image segment

1. Use the navigation bar at the top of the image window.

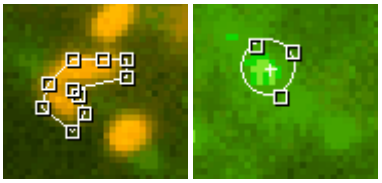


2. Move the slide control slowly, and by doing so display frames acquired at differing Z-positions in the image window. Search out a Z-position at which the sample can be clearly recognized.
3. Use the *View > Toolbars > Life Science Application* command, to have the *Life Science Application* toolbar displayed. You can find the functions for defining ROIs and for measuring the intensity profile on this toolbar.

Defining ROIs (Region Of Interest)

1. Rotate the mouse wheel to change the zoom factor. Enlarge the image until you can see at least one enlarged segment of the sample in the image window that is fluorescing in red.

2. Click the *New ROI - Polygon* button on the *Life Science Applications* toolbar.
3. By clicking with your left mouse button, define an area on the image that only includes red fluorescing sample positions.
4. Right click to finish the definition of the ROI.
5. Then define another ROI on an image segment that only includes green fluorescing sample positions.



6. Click the *New ROI - Rectangle* button.
7. Define a square in a dark image segment that shows no fluorescing objects. This ROI will be used as a reference for the background correction.

Computing intensity profiles

1. On the *Life Science Applications* toolbar, click the *Intensity Profile* button.
   - The *Intensity Profile - <Name of the active image>* dialog box opens.
   - Your software recognizes the image type, and automatically selects the corresponding option. In this example the *Z-profile* option is preset.
2. Select the *Results > Average* check box.
   Clear all the other check boxes.
   - In the *ROI data* group, all of the ROIs that have been defined on the active image will be listed. In this example, you'll find three ROIs there (two on sample positions showing different fluorescence colors and one on the background).
3. Each ROI defines a specific image segment. Now, select the image segments for which intensity profiles are to be calculated.

   In this example, select both of the ROIs at fluorescing sample positions.
4. In the *Background Subtraction* group, select the *ROI* option.

- In the list next to the *ROI* option, all of the ROIs that have been defined on the active image will be listed.

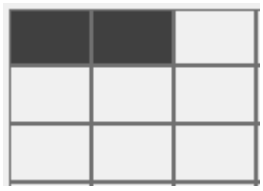
5. In the list, select the ROI that was defined on the image background.
6. Click the *Execute* button.
   - The intensity profiles will be calculated and displayed in the *Intensity Profile* tool window.

Viewing intensity profiles

1. If necessary, use the *View > Tool Windows > Intensity Profile* command, to show the tool window. The tool window offers you several ways of displaying the intensity profile that has been measured.
2.  In the *Intensity Profile* tool window's toolbar, click the *Arrange intensity profile charts* button.
   - The *Arrange intensity profile charts* dialog box opens.
3. Make the following settings in the dialog box.

   Select the *Select all* check box, in the *Show charts* group.

   In the *Layout* group, specify a grid size of 2x1.

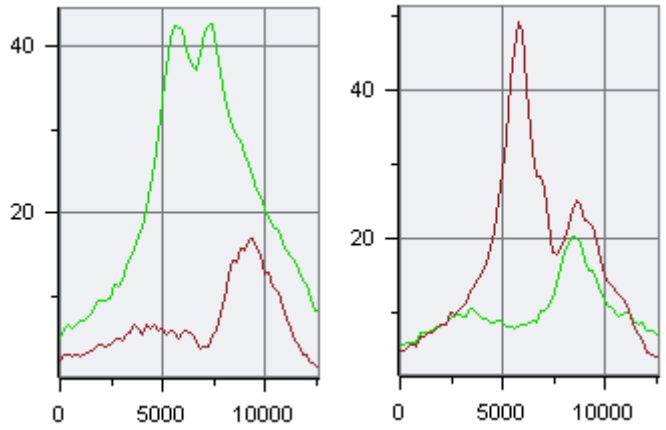
   

   Close the dialog box with *OK*.
4.  In the *Intensity Profile* tool window's toolbar, click the *Arrange intensity profile data* button.
   - The *Arrange intensity profile data* dialog box opens.
5. Make the following settings in the dialog box.

   Select the *Separate chart per channel* check box.

   Clear the other check boxes.

   Close the dialog box with *OK*.
   - You can see two charts, each with two intensity profiles. Along the X-axis the Z-position, that's to say, the height, has been plotted. The intensity is plotted on the Y-axis.



For each of the image's color channels, an individual chart will be created. The name of the corresponding color channel will be displayed in the chart's header. On the left, you see the results for the green color channel, on the right, those for the red one.
In each chart, you can see an intensity profile for each ROI that has been defined. You can display a legend with the name of the ROIs in the chart. The green intensity profile was measured on the ROI on the green fluorescing position on the sample, the red on the red fluorescing position.

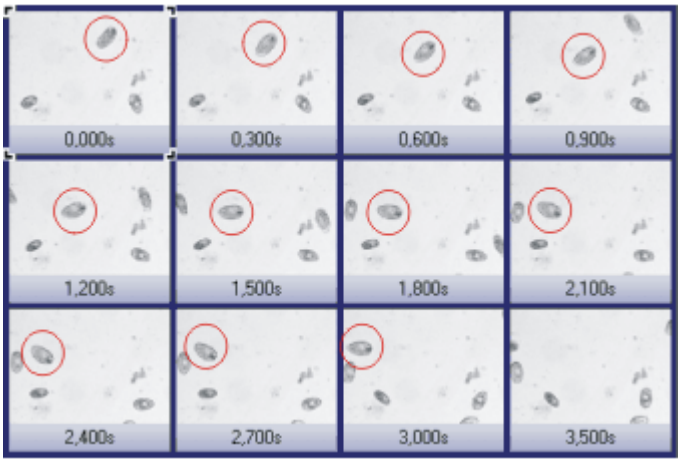
Exporting and saving intensity profiles

1. In the *Intensity Profile* tool window's toolbar, click the *Export to Workbook* button.
   - A new workbook will be created in the document window. This workbook contains results sheets with all of the results.

     When you've measured a multi-channel image, you'll find an individual work sheet for each of the color channels.
2. Use the *File > Save As* command to save a workbook.
   - A workbook will be saved in the OWB file format. This format is an exclusive file format and can only be opened with your software. Workbooks are, obviously, therefore not suitable for using to exchange data with other application programs. If you would like to use the results in a different application, use the *File > Export to > Excel* command.

10.1.3. Measuring the intensity profile of moving objects

Task: You've acquired a time stack of moving paramecia. Define a dynamic ROI that contains a paramecium and move the ROI so that it follows the paramecium through all of the frames in the time stack.
Measure the intensity profile.



The illustration shows an overview over the frames in a Z-stack. The time points associated with the frames are shown under the images. The red circle shows the movement of a single paramecium.

1. Several example images were supplied together with your software. You can follow these step-by-step instructions using the ParameciumTimeSeries.tif example image.

Specifying the user interface and default settings

2. Use the *View > Toolbars > Life Science Application* command, to have the *Life Science Application* toolbar displayed. You can find the functions for defining ROIs and for measuring the intensity profile on this toolbar.
3. Use the *View > Tool Windows > Measurement and ROI* command to display the *Measurement and ROI* tool window. The ROIs that are defined in the current image are listed in this tool window.
4. Use the *Tools > Options* command. Select the *Measurement and ROI > Dynamic ROI* entry in the tree view.

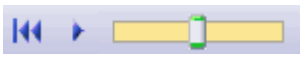
   Select the *Interpolate linearly, continue with current* option.

   You have now defined how a dynamic ROI behaves when you define its position on the frames.

   Close the dialog box with *OK*.

Viewing the movement of the paramecia

5. Use the navigation bar at the top of the image window.



6. Move the slide control slowly to the right to view the movement of the paramecium that is at the top left border of the image in the first frame.
   - The paramecium first moves down and to the left. Then it changes direction and moves up before finally disappearing at the left border of the image.

Defining ROIs (Region Of Interest)

7. Display the first frame in the image window. To do this, use the navigation bar at the top of the image window.

8. Click the *New ROI - Rectangle* button on the *Life Science Applications* toolbar.
   - The ROI is displayed in the sheet of the *Measurement and ROI* tool window. In the *Type* column, the keyword *(ROI)* is added to the type name.
9. With two mouse clicks, define a small rectangle around the paramecium at the top left border of the image.

10. Move the mouse pointer over the ROI you just defined. Click the right mouse button to open a context menu. Select the *Convert to dynamic ROI over t* command from the context menu to turn the static ROI into a dynamic ROI.
    - In the *Measurement and ROI* tool window, the keyword *(ROI)* in the *Type* column changes into the new keyword *(dROI [t])*.

Following the movement of the object using the dynamic ROI

11. In the image window, display the frame in which the paramecium changes its direction.
    - The position of the ROI that has been defined is the same on all frames.
12. Move the ROI on this frame so that it contains the paramecium again.
    - The system will now automatically reposition the ROI on each frame between the first and the current frame. The positions are calculated as a linear interpolation of the ROI positions in the first and the current frames. Check whether the paramecium is completely within the ROIs on these frames.
13. In the image window, display the last frame in which the paramecium is still completely visible in the image.
14. Move the ROI on this frame again so that it contains the paramecium again. Make sure that the ROI doesn't include any other paramecium.
15. Check whether the ROIs position is correct for all of the frames up till now.
    - In the following frames, the paramecium disappears at the left border of the image and so can't be measured any more. The dynamic ROI is still defined on all following frames in the image series. You can't delete a dynamic ROI only for particular frames.

Calculating an intensity profile

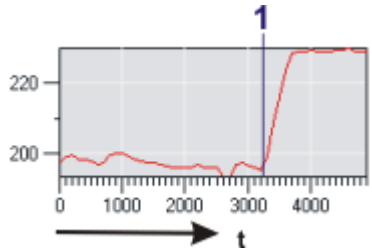
16. On the *Life Science Applications* toolbar, click the *Intensity Profile* button.
    - The *Intensity Profile - <Name of the active image>* dialog box opens.
    - Your software will recognize the image type, and will select the appropriate option in the *Method* group. In this example, the *Over time* option is preset.
17. Make the following settings in the *Intensity Profile* dialog box.

    Select the *Results > Average* check box.

    Clear the other check boxes.

    Select the dynamic ROI in the *ROI data* group.

    In the *Background subtraction* group, select the *none* option.
18. Click the *Execute* button.
    - The intensity profile for the paramecium will be calculated and displayed in the *Intensity Profile* tool window.



The intensity profile displays how the average intensity in the ROI changes over time. The ROI contains the paramecium until about 3000 ms. The intensity is relatively constant.
At time point (**1**), the paramecium begins to leave the image. The intensity then increases to the level of the light image background. The time point is at about 3300 ms.

10.1.4. Displaying intensity profiles in the tool window

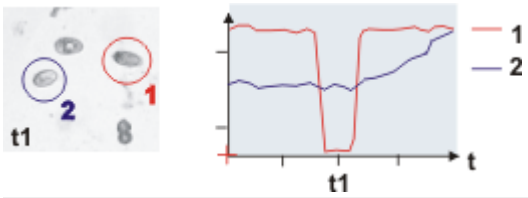
Computing intensity profiles on more than one image segment

Compute the intensity profiles for several image segments on a time stack. Display the intensity profiles in a single chart. Change the arrangement of the intensity profiles by creating a separate chart for each intensity profile.

1. Load or acquire a monochrome time stack.
2. Define several image segments (ROIs) on the image.
3. Use the *Measure > Intensity Profile...* command to open the *Intensity Profile* dialog box.
4. Select all of the ROIs in the *ROI data* list in the *Intensity Profile* dialog box. Click on every ROI that isn't highlighted to do this.
5. Select the *Results > Average* check box to compute the average intensity value in the image segment. Clear the check boxes in the *Results over all ROI* group.
6. Click the *Execute* button to create an intensity profile for each ROI.
   - The intensity profiles are displayed in the *Intensity Profile* tool window.
   - The intensity profiles are displayed in the same color as their ROIs by default.

Displaying intensity profiles together in the same chart

7. Click the *Arrange intensity profile data* button. You can find this button on the *Intensity Profile* tool window's toolbar.
8. Select the *Separate chart per measurement (Average, Min, Max, Integral)* check box in the *Arrange intensity profile data* dialog box. Clear all the other check boxes.
   - In the *Intensity Profile* tool window's chart area, each ROI that has been defined has its own intensity profile. The color of the intensity profiles corresponds to the color of the ROI. The chart is titled *Average*.



The illustration shows an example of intensity profiles over two image segments.
On the time stack on the left, two ROIs have been defined. Here, only the frame of time point t1 is shown.
On the right, the intensity profile has been measured against the time, for each ROI. For each ROI the mean intensity value within the ROI is plotted.
The red intensity profile belongs to ROI1. You can clearly see that the dark cell in ROI1 moved in and back out of the ROI very quickly. The intensity profile has a clear minimum at time point t1, since only at this time point did the dark cell (low intensity values) almost fill out the complete ROI. At every other time point there was only bright background (high intensity values) within the ROI. By contrast, the cell in ROI2 didn't move as quickly. The blue intensity profile doesn't show any pronounced minimum.

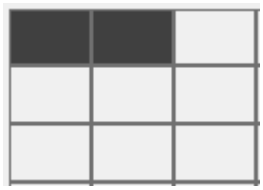
Displaying each intensity profile in its own chart

9. Click the *Arrange intensity profile data* button again.
10. Select the *Separate chart per ROI* check box in the *Arrange intensity profile data* dialog box. Clear all the other check boxes.

    Close the dialog box with *OK*.
11. Click the *Arrange intensity profile charts* button. You can find this button on the *Intensity Profile* tool window's toolbar.
12. Make the following settings in the dialog box.

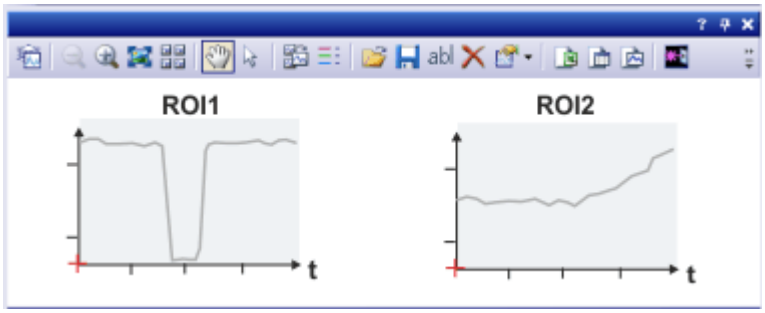
    Select the *Select all* check box, in the *Show charts* group.

    In the *Layout* group, specify a grid size of 2x1.

    Close the dialog box with *OK*.

    - The intensity profiles in the *Intensity Profile* tool window are now arranged differently. You now see a separate chart for each ROI that was defined. The two charts are positioned next to each other. The titles of the charts correspond to the names of the ROIs.



The *Intensity Profile* tool window contains two charts. They are positioned next to each other.

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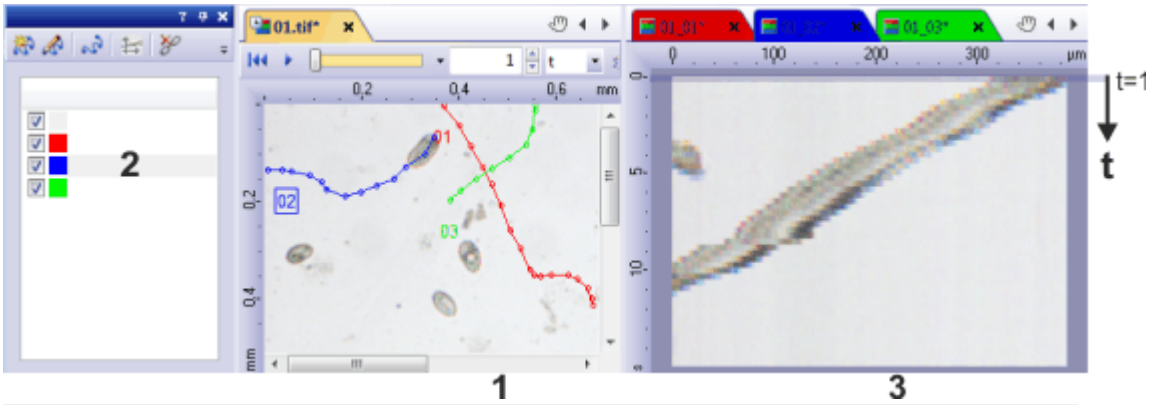
10.2. Kymograph

10.2.1. Overview - Kymograph

Use the *Kymograph* tool window to create a visual representation of the movement of objects in an image series.

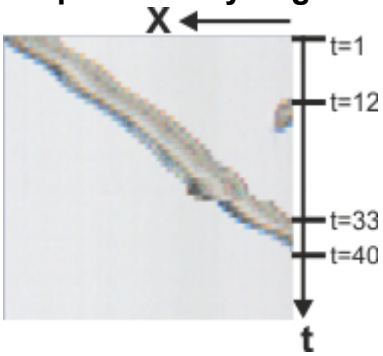
What does the kymograph measure?

Define one or more tracks on an image series. A track is a line that can follow any course you want. You can assign a particular width to it. For each track, the kymograph computes the intensity values along the line and plots these values against the time or the Z-value. The result is one kymogram for each defined track. The kymogram is an image that is calibrated differently on the horizontal and vertical axes. With a time stack for example, a kymogram's X-direction is calibrated in units of length and the Y-direction is calibrated in units of time.



In the above example, three tracks are defined on the image series (**1**). The kymogram is computed for each track. The kymograms are automatically arranged to the right of the image series.
Each of the tracks has been assigned a different color. The header of the corresponding kymogram in the document group has the same color.
In the illustration, the blue track (**2**) is selected in the tool window. This displays the corresponding kymogram (**3**) in the document group to the right of the tool window. The topmost line in the kymogram shows the intensity profile along the blue line, the track, at time point t=1. The dark object is at the very right of the track at this time point. The kymogram shows that the object keeps moving further to the left.

Properties of kymograms



The illustration shows a kymogram that has been computed from a time stack in which dark objects move across a light background.

The intensity values along a line in the image are plotted along the image's X-direction. The line has been defined to follow an object's track precisely. The time is plotted along the Y-direction.
At time point **t=1** the object is right at the start of the track.
At time point **t=12** an additional object enters the image.
At time point **t=33** the object starts to move out of the image.
After time point **t=40** no object is visible on the defined track.

Saving the results

You can save a kymogram like a normal image. Use the VSI or TIF image format to preserve the calibration.

When you save an image series that has tracks defined on it, the tracks are saved together with the image. Use the *Kymograph* tool window to re-compute the kymograms from the tracks at the press of a button.

Making measurements on a kymogram

Use the *Kymogram Polyline* measurement function to make measurements on a kymogram.

The remaining interactive measurement functions in the *Measurement and ROI* tool window cannot be used for the measurement of kymograms.

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10.2.2. Visual representation of periodic movement

Task: You have acquired a multi-channel time stack. The intensity of the fluorescence constantly rises and falls within the sample. You want to create a visual representation of the intensity profile.

The following process flow chart displays the basic steps of the process.

Creating a new track

Load an image series that contains moving objects.
Create an entry for a new track in the *Kymograph* tool window.

Defining a track

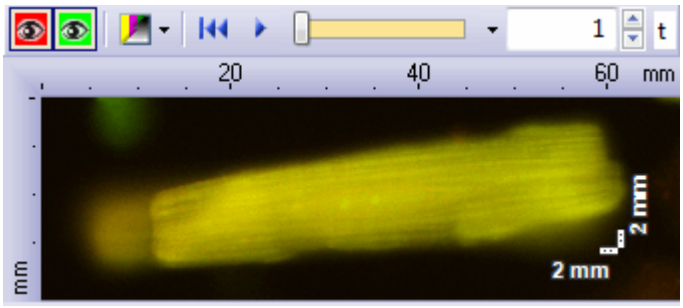
Define the course of the track on a projection image of the image series.

Computing a kymogram

Compute the kymogram for the defined track.

Preparing the analysis

1. Load the image series that you want to analyze.
   - When you load a multi-channel time stack, it will be automatically displayed in the Single Frame View in the image window.
2. When working with the kymograph you should display horizontal and vertical scale bars in the image. To do this, use the *Tools > Options* command. Select the *Scale Bar > Display* entry in the tree view. Select the *Orientation > Horizontal and vertical* option.
3. If the scale bars aren't displayed in the image window, select the *View > Scale Bar* command to show them.



The illustration shows the first image of a multi-channel time stack.

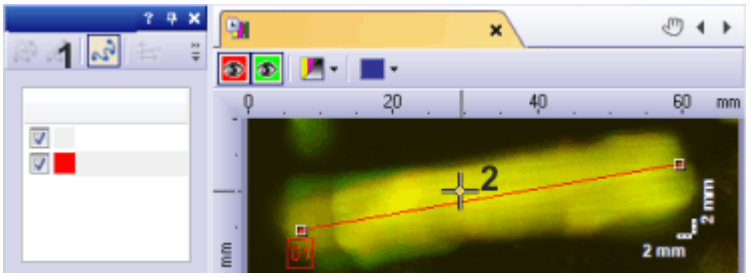
4. Use the *View > Tool Windows > Kymograph* command to make the *Kymograph* tool window appear.

Creating a new track

5. Click the *Create Track* button in the *Kymograph* tool window to create a new track on the active image series.
   - The *Create Track* dialog box opens.
6. Enter a name for the track in the *Track definition > Name* field, *Heart_Muscle-01* for example.
   In the *Color* field select a color that is easy to see on the image, red for example.
   In the *View* list, check whether the correct projection method is being used. In this example, because the moving object is light and the background is dark, select the *Maximum intensity projection* entry from the list.
   Close the *Create Track* dialog box with *OK*.
   - A new entry is created in the *Tracks* list in the *Kymograph* tool window.

Defining a track

7. Click the *Define Track Polyline* button in the *Kymograph* tool window.
   - The image series is automatically displayed in the *Maximum Intensity* projection view in the image window.
   - The mouse pointer jumps to the image window and turns into a cross.
   - All of your software's other functions are now blocked.



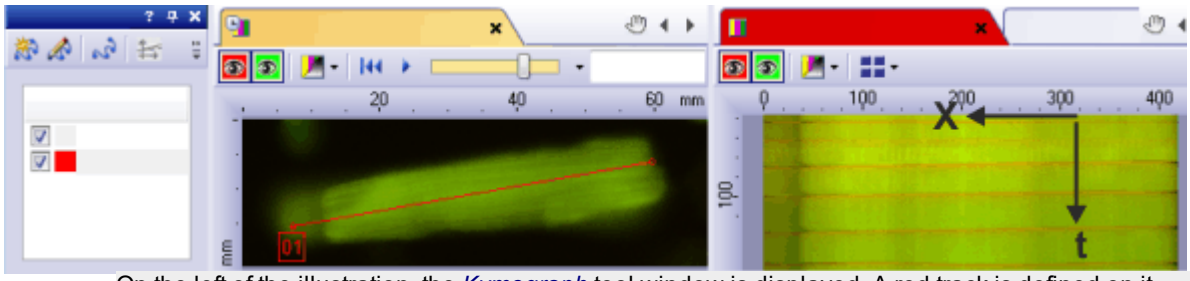
The *Define Track Polyline* (**1**) button appears clicked to indicate the current mode. Define the track (**2**) on the image series' projection view.

8. Define the track by left clicking.
9. Right click to finish the definition of the track.

Computing a kymogram

10. Click the *Compute Kymogram* button in the *Kymograph* tool window to compute the kymogram for the defined track.
    - The kymograph computes the intensity values along the course of the track and plots these values against the time. The result is a kymogram. The kymogram is a normal image that is calibrated differently on the horizontal and vertical axes.
    - The kymogram is automatically arranged to the right of the source image.
    - The kymogram's name is made up of the image's name plus the name of the track.

- The header of the kymogram in the document group is the same color as the track.
- The kymogram is computed separately for each color channel. Use the color channel buttons in the navigation bar to view the kymograms for the individual color channels.

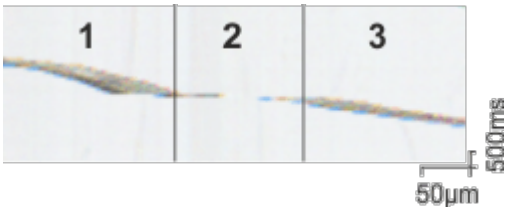


On the left of the illustration, the *Kymograph* tool window is displayed. A red track is defined on it. You can see the time stack in the center. In this case it's a multi-channel time stack. The red track runs straight across the object. This object is muscle tissue.
The kymogram on the right clearly shows a periodic movement of the object. The tissue contracts and expands. A coordinate system with the image dimensions is shown on the kymogram. The width of the kymogram is defined by the length of the track. The height of the kymogram is determined by the number of frames in the time stack.

Saving the results

11. Activate the image series in the document group. Select the *File > Save As* command and save the image series together with the defined tracks. Use the TIF or VSI image file format.
12. Activate the kymogram in the document group. Select the *File > Save As* command and save the kymogram as an image file. Use the TIF or VSI image file format.

10.2.3. Making measurements on a kymogram



The illustration shows a kymogram that has been computed from a time stack in which dark objects move across a light background. The movement can be roughly divided into three phases (**1**-**3**). In phases (**1**) and (**3**), the object is moving at a similar speed. It needs about the same time to cover a distance of 150 µm, for example. The object moves considerable faster in phase (**2**).

Task: Measure the speed of the object on the above example image in phases **1**-**3**.

1. Load the kymogram you want to measure or create a new one.

Defining a measurement object

2. Click the *Kymogram Polyline* button on the *Measurement and ROI* toolbar.
   - Your software will automatically switch to the measurement mode. Your mouse pointer appears in the image window as a cross.

- The selected measurement function is displayed to the bottom right of the mouse pointer.

3. Define the polyline on the kymogram by left clicking the mouse button. Click along the track of the moving object.
   - Your software connects two neighboring control points with a straight line.
   - Each click defines a segment of the polyline. The measurement results deliver measurement values that refer specifically to these segments.

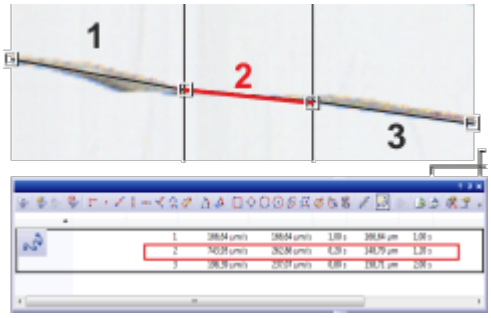
Note: If possible, define the polyline without any overlap or intersections.

4. Right click to finish the definition of the measurement object. Note that the last click also defines a segment of the polyline.

A measurement object (in red) has been defined on the kymogram. The measurement object is a polyline that has been defined with the control points precisely on the borders of the object's movement phases.

Viewing the measurement results

5. Use the *View > Tool Windows > Measurement and ROI* command to display the *Measurement and ROI* tool window.
   - In the table in the *Measurement and ROI* tool window, a new measurement value of the *Kymogram* type will be entered.
   - Note that several segments belong to the measurement object that was measured. Each segment has its own measurement values. In the table in the *Measurement and ROI* tool window, several measurement values have been assigned to a single entry in the *Type* or *Name* column.



You can view the measurement results in the *Measurement and ROI* tool window after the measurement has been performed. You receive a measurement object with measurement values for each segment that was defined. The speed of the object in phase 2 can be found in the row belonging to segment 2.

Selecting a measurement parameter

Your software offers a wide range of measurement parameters for making measurements on a kymogram. You should now check whether the measurement parameters that interest you are also being displayed in the *Measurement and ROI* tool window.

6. In the *Measurement and ROI* tool window, click the *Select Measurements* button.
   - The *Select Measurements* dialog box opens. In the dialog box, at the top left, you'll see a list with all of the available measurement parameters. At the bottom of the dialog box, you'll see a list of the measurement parameters that are currently calculated and displayed for all objects.
7. In the *Available measurements* list, click the *Object Type* column header.
   - Measurement parameters that can be used on a kymogram are of the *Kymogram Line* object type.
8. In the *Available measurements* list, select a measurement parameter of the *Kymogram Line* type, *Current Velocity* for example.

   - The large button beneath the list of available measurements shows the names of the selected measurement parameters.

9. Click the *Add 'Current Velocity'* button to have the measurement parameter added to the list of calculated measurement parameters.
   - This measurement parameter will be added to the list of measurement parameters to be calculated. All these measurement parameters will be shown in the *Measurement and ROI* tool window.
   - Add all of the measurement parameters that you want to the list of computed measurement parameters.
   - You should definitely add the *Segment ID* measurement parameter to the results table. It will enable you to correlate the measurement values with the segments that were defined.
11. Close the *Select Measurements* dialog box with *OK*.
   - The results table in the *Measurement and ROI* tool window now displays the selected measurement parameters.

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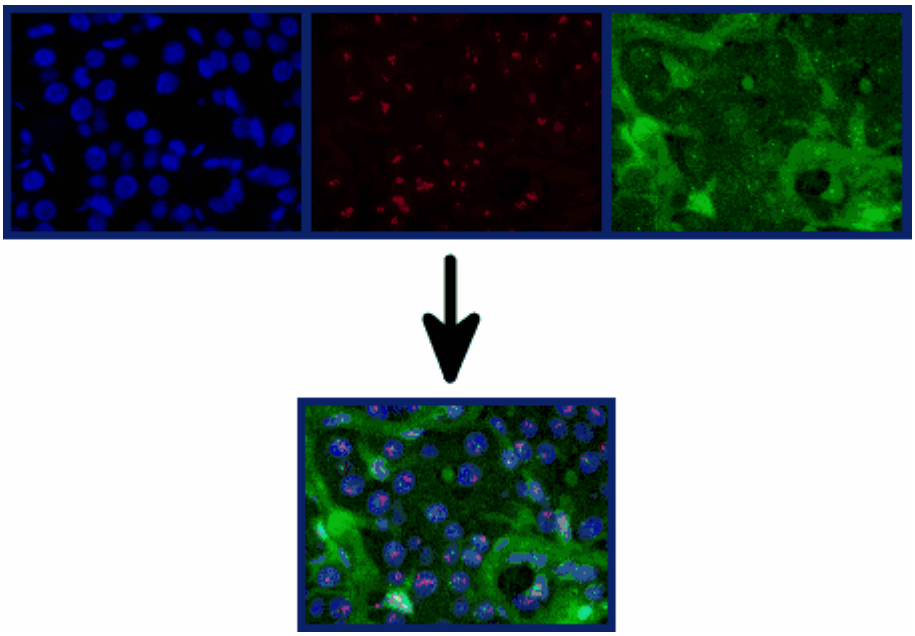
10.3. Fluorescence Unmixing

10.3.1. Overview - Fluorescence Unmixing

Multi-channel fluorescence microscopy

In the multi-channel fluorescence microscopy, different cell structures will be visually separated by acquiring them separately, then displaying them in different colors. To achieve this, one stains the sample with several suitably chosen fluorochromes. Each of these labels a special cell structure. The fluorescence images will then be acquired. The fluorescence image 1, created with fluorochrome 1 shows cell structure 1, the fluorescence image 2 created with fluorochrome 2 shows cell structure 2, etc.. The individual images will be combined into a multi-channel fluorescence image that shows the different cell structures in different colors. When, for example, three fluorochromes are used, a three channel fluorescence image will be created.



Problem with the visual separation of the structures

Filter sets in the microscope
Your microscope has an appropriate filter set for each fluorochrome, this set comprises an excitation filter, a dichromatic mirror, and an emission filter. When the fluorochrome 1 is excited by light from the wavelength range 1a, it emits light in the wavelength range 1b. When fluorescence image 1 is acquired, the excitation filter 1 takes care that only light from a narrow range within the wavelength range 1a reaches the sample from the microscope's illumination source. At the same time, the dichromatic mirror 1 and the emission filter 1 take care that, from the sample, only light from a narrow range within the emission wavelength range 1b, reaches the camera.

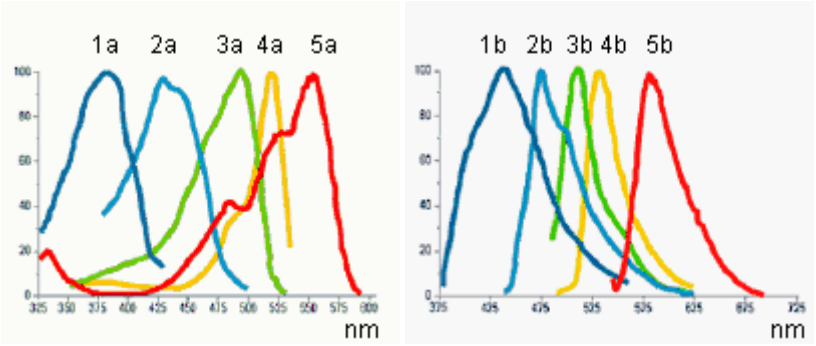
Overlapping of the wavelength ranges

The problem with this procedure is, that the wavelength ranges of the different fluorochromes overlap. If this overlapping didn't occur, the aspired visual separation of the different cell structures in the resulting multi-channel fluorescence image would be perfect.

Neither the excitation wavelength ranges nor the emission wavelength ranges have sharp limits, and they lie very close to one another, where numerous fluorochromes are concerned. Therefore, the excitation wavelength ranges 1a, 2a, 3a, ... of the fluorochromes 1, 2, 3, ... normally overlap. The same applies to the emission wavelength ranges 1b, 2b, 3b ... As well as that, there are also overlappings between excitation wavelength ranges and emission wavelength ranges.

Excitation and Emissions spectra

In the spectra that follow, you can see a graphical demonstration of the way in which the excitation intensities and the emission intensities of several fluorochromes that are often used, depend on the wavelength. The way in which the different wavelength ranges overlap, can clearly be seen in these spectra.



Spectral unmixing

Owing to the spectral overlapping, the aspired visual separation of the different cell structures only succeeds partially. When, for example, the light that excitation filter 1 lets through, also excites fluorochrome 2 a little, and part of the light that fluorochrome 2 then emits can pass the emission filter 1; cell structure 2 will also be dimly visible in fluorescence image 1. One can then speak of an unwanted "spectral mixing" of the individual fluorescence images.

Spectral unmixing

The spectral mixing can be subsequently removed from a digitally recorded multi-channel fluorescence image, by recalculation. That's to say, the image will be "spectrally unmixed". When you do this, it improves the visual separation of the different cell structures in the image, and improves the image quality. To do that, use the *Process > Enhancements > Fluorescence Unmixing* command.

The spectral unmixing of a multi-channel fluorescence image takes place in two steps. The first step is the calibration of the color channels with the help of reference images. When the experimental conditions don't change, you will only need to carry out this step once. In the second step, the actual spectral unmixing takes place.

You require precisely one reference image for each color channel that is to be calibrated. Each reference image must have exactly the same number of color channels as the image that is to be unmixed. In the instructions that follow, it will be assumed that you have acquired a three channel fluorescence image and want to carry out a spectral unmixing with it. For a two channel fluorescence image the procedure is analogical.

10.3.2. Calibrating color channels

When the experimental conditions don't change, you will only need to carry out the calibration once. When you've done that, you can spectrally unmix all of the three channel fluorescence images that are acquired later, on the basis of this calibration.

Acquiring reference images

1. Set up three samples that in each case have only been stained with one of the three fluorochromes.
   Alternatively, you can use a single sample that has been stained with all three fluorochromes. In this case, there must be three areas on the sample that have each been stained with only one fluorochrome.
2. Acquire a three channel fluorescence image of each of the three samples (alternatively, of each of the three areas on your sample).
   When you do this, use either the excitation filter appropriate for each of them, and a multiband emission filter or a multiband excitation filter and the emissions filter appropriate for each of them.
   The experimental conditions must be the same as they were when the image that is to be spectrally unmixed was acquired.
   - Differences in the exposure times of the individual color channels will be automatically linearly corrected when a spectral unmixing is carried out. Nevertheless, as a rule it makes sense not to change the exposure times when the reference images are acquired.
   - The result will be three multi-channel fluorescence images. Each of them contains three channels. These will, in what follows, be designated as "reference image 1", "reference image 2" and "reference image 3". Reference image 1 is to be used to calibrate color channel 1, that belongs to fluorochrome 1. With the reference images 2 and 3 the method is analogical.
   - Each of the reference images will be displayed in its own window, in the document group. The reference image that was last acquired will be the currently displayed, active image.
   - Should you have already acquired the three reference images at an earlier point in time, you can load them into the document group by using the *File > Open > Image* command.

Defining ROIs

1. Activate reference image 1 in the document group.
2.  In the *Life Science Applications* toolbar, click the *New ROI - 3 Point Circle* button.

- Should the toolbar not be visible, put it on display by using the *View > Toolbars > Life Science Applications* command.

3. Search out an area in reference image 1, in which fluorochrome 1 is especially bright and glows as evenly as possible.
4. Define a circular ROI within this area with three mouse clicks.
   - This ROI was defined for the fluorochrome 1. It will be automatically assigned the name "ROI 1".
   - You can still subsequently change the size and position of this ROI.
   - You can change this automatically created name. To do so, use the *Measurement and ROI* tool window. In it, click the ROI's name to change it. Should the tool window not be visible, put it on display by using the *View > Tool Windows > Measurement and ROI* command.
5.  Click the *New ROI - 3 Point Circle* button once more.
6. Search out a dark area in the background of reference image 1, in which, as far as possible, no fluorochrome can be seen.
7. Define a circular ROI within this area with three mouse clicks.
   - This ROI was defined for the image background. It will be automatically assigned the name "ROI 2".
8. Using the same procedure, define in reference image 2 an ROI for the fluorochrome 2, and an ROI for the image background.
9. Using the same procedure, define in reference image 3 an ROI for the fluorochrome 3, and an ROI for the image background.

Finishing the calibration

1. Activate reference image 1 in the document group.
2.  In the *Life Science Applications* toolbar, click the *Fluorescence Unmixing* button to open the *Fluorescene Unmixing* dialog box.
3. Activate the *Calibration* tab.
4. Enter the label for fluorochrome 1 in the *Name* field. This is, at the same time, the name for the calibration for color channel 1.
5. Select reference image 1 in the *Image* list.
6. In the *ROI* list, located immediately below the *Image* list, select "ROI 1" which was defined for the fluorochrome 1.
7. In the *Background subtraction* group, select the *ROI* option for the background correction of reference image 1.
8. In the neighboring list to the right, select "ROI 2" that was defined for the image background in reference image 1.
9. Click the *Save* button.
   - The calibration of color channel 1, has now been completed.
   - The *Fluorochrome 2 >>* button will become available.
10. Click the *Fluorochrome 2 >>* button to skip to the calibration of color channel 2.
    - The name of the *Fluorochrome 1* group will then change to *Fluorochrome 2*.

11. Then, using the same procedure, calibrate color channel 2 with reference image 2 and fluorochrome 2.
12. Then, using the same procedure, calibrate color channel 3 with reference image 3 and fluorochrome 3.
13. Click the *Cancel* button, to close the *Fluorescence Unmixing* dialog box.

10.3.3. Spectrally unmixing a three channel fluorescence image

1. In the document group, activate the three channel fluorescence image you want to spectrally unmix.
   - Should you have already acquired the image at an earlier point in time, you can load it into the document group by using the *File > Open > Image* command.
2.  In the *Life Science Applications* toolbar, click the *New ROI - 3 Point Circle* button.
3. Search out a dark area in the background of your image, in which, as far as possible, no fluorochrome can be seen.
4. Define a circular ROI within this area with three mouse clicks.
   - This ROI was defined for the image background. It will be automatically assigned the name "ROI 1".
5.  In the *Life Science Applications* toolbar, click the *Fluorescence Unmixing* button to open the *Fluorescene Unmixing* dialog box.
6. Activate the *Linear Unmixing* tab.
7. In the *Fluorochrome 1* list, select the calibration with which the fluorescence image in your multi-channel fluorescence image's color channel 1 is to be corrected.
   - The name of this calibration is identical with the label you gave the fluorochrome 1 while you were performing the calibration.
8. In the *Fluorochrome 2* list, select the calibration with which the fluorescence image in your multi-channel fluorescence image's color channel 2 is to be corrected.
9. In the *Fluorochrome 3* list, select the calibration with which the fluorescence image in your multi-channel fluorescence image's color channel 3 is to be corrected.
10. In the *Background subtraction* group, select the *ROI* option for the background correction of your image.
11. In the neighboring list to the right, select "ROI 1" which was defined in your image for the image background.
12. Click the *OK* button to carry out the spectral unmixing and to close the dialog box.
    - A new image document will be created for the spectrally unmixed image. The source image will not be changed.
    - It can occur that, immediately after the spectral unmixing, the image will not be optimally displayed on your monitor. In this case, click the *Apply* button in the *Adjust Display* tool window. When you do this, the image contrast on your monitor will be automatically optimized. The actual image data will not be changed.

13. Save the spectrally unmixed image if you need it.

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10.4. Measuring the colocalization

Use the *Colocalization* button to start a measurement of colocalization. You can find this button on the *Life Science Applications* toolbar.

Prerequisite: This button isn't available in all software versions.

What is a measurement of colocalization?
In fluorescence microscopy, you examine samples that have been stained with different fluorochromes. The measurement of colocalization determines on which areas of the sample fluorescence signals from two different fluorescence channels are simultaneously present. A measurement of colocalization results in a scatterplot, a colocalization image, and different measurement parameters.

10.4.1. Measuring the colocalization on the whole frame

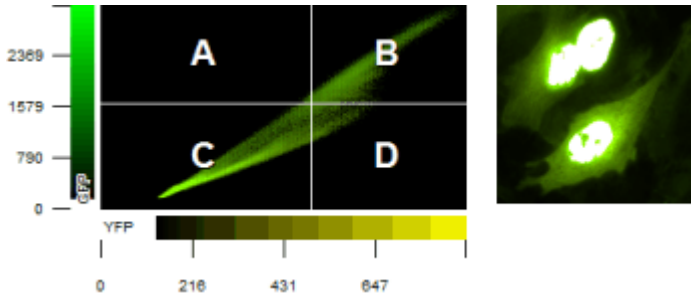
1. Load the multi-channel image you want to use for the colocalization measurement.
2.  On the *Life Science Applications* toolbar, click the *Colocalization* button. If this toolbar is not displayed, use the *View > Toolbars > Live Science Applications* command.
   - The *Colocalization* dialog box opens.
3. From the *Scatterplot > Channel 1* and *Channel 2* fields, select the two color channels on which you want to perform the measurement of colocalization.

Selecting the preview function

4.  In the *Preview* group, click this button . Select the *Target channels and colocalization channel* command from the menu.
   - The active preview function is identified by a check mark.

Selecting the intensity range in the scatterplot

5. In the *Target area* group, select the *Whole frame* entry from the *Area* list.
6. In the scatterplot, define the intensity range that you want the measurement of colocalization to analyze. To do so, select, for example, the *Mode > Threshold* option.
   - A grid appears in the scatterplot, dividing it into quadrants.
7. Select the *B (upper right)* entry in the *Use quarter* list. Now the colocalization channel will include all of the pixels that have a high intensity in both channels. Notice how the preview changes.
   - In the preview, the pixels whose intensity values are within the quadrant that has been selected are shown in white.



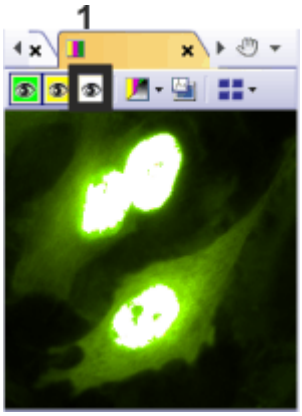
The illustration on the **left** shows a scatterplot in which the pixels are located more or less along a diagonal line. That's to say, the pixel intensity values are in both fluorescence channels approximately the same. In the resulting image on the **right**, all of the pixels whose intensity is in the B quadrant are shown in white. This enables you to quickly identify the image areas in which both channels have particularly high fluorescence intensities.

Selecting frames for the measurement of colocalization

8. When you work with multi-channel time stacks or multi-channel Z-stacks: Determine in the *Apply on* group, whether the colocalization measurement is to be carried out on all frames or only on selected frames. Should you want to limit the image selection, select the *Selected frames* entry, then click the *Dimension Selector* button.
   - Then you can limit the image selection in the *Dimension Selector* tool window.
9. Use the navigation bar in the image window to browse through the image series. Select the frame for which you want to determine a scatterplot.

Viewing the results

10. In the *Colocalization* dialog box, you can see the results for the current frame in the image series in the preview and in the *Result (current frame, all ROIs)* group. Please note that you can change the size of the dialog box. You can double click the dialog box's header to enlarge the dialog box to full-screen size.
11. Click the *Options* button, then select the *Colocalization channel (Image)* and *Measurement results (Workbook)* check boxes. Close the dialog box with *OK*.
12. Click the *OK* button in the *Colocalization* dialog box to finish the measurement of colocalization.
    - A workbook that contains the results of the colocalization measurement will be created. The workbook contains two worksheets.
      The first worksheet, *Colocalization <Channel 1>/<Channel 2> of <Name of the source image>*, displays the measurement results for the selected quadrants. The *Pearson's Correlation Coefficient R(r)* was measured, for example.
      The second worksheet, *<Threshold>*, displays measurement results for all of the quadrants. The number of pixels in the quadrants has been measured.
    - A new image that contains the colocalization channel will be created. In the navigation bar, you can find a separate button, in the corresponding fluorescence color, for each of the image's color channels. The colocalization channel is white. Use this button to show or hide the colocalization channel.



The colocalization image appears as an additional channel in the source image. Click the (**1**) button to hide the colocalization image.

13. If required, use the *File > Save As* menu command to save the new image and the workbook.

10.4.2. Measuring the colocalization on a part of the image (ROI)

Frequently, a colocalization of fluorescence signals occurs only in a small image segment. In this case, it makes sense to define a ROI (Region of Interest) then determine the colocalization only within this ROI. You can also define several ROIs. ROIs can have any shape you wish.

1. Load the multi-channel image you want to use for the colocalization measurement.
2. On the *Life Science Applications* toolbar, click the *Colocalization* button.
3. From the *Scatterplot > Channel 1* and *Channel 2* fields, select the two color channels on which you want to perform the measurement of colocalization.

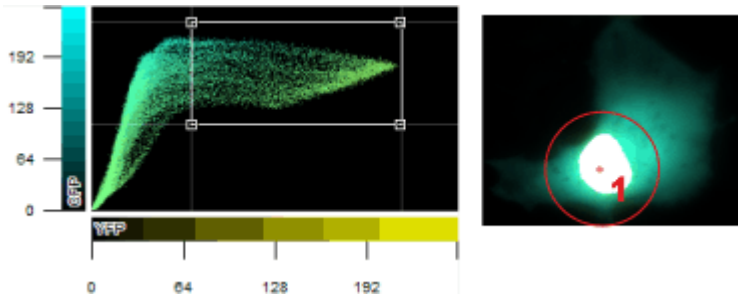
Selecting the preview function

4. In the *Preview* group, click this button . Select the commands *Target channels and colocalization channel* and *Show target area* from the menu.
   - The active preview functions are identified with a check mark.
5. In the scatterplot, define the intensity range that you want the measurement of colocalization to analyze. To do so, select, for example, the *Mode > Rectangle* option.
   - A rectangle will appear in the scatterplot.
   - In the preview, the pixels whose intensity values are within the rectangle that has been defined are shown in white.
6. If required, use the mouse to move the rectangle in the scatterplot.

Defining ROIs

7. In the *Target area* group, select the *ROI* entry from the *Area* list.
   - Next to the field, to the right, the buttons with the various ROI forms are displayed.

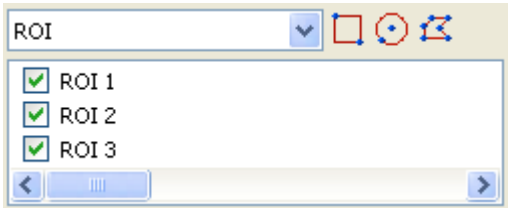
8. Click the button for the required ROI form that you want to set up. You have the choice between a rectangle, a circle and a polygon.
   - The mouse pointer will appear in the image window. The *Colocalization* dialog box is hidden.
9. Define the first ROI with clicks of your left mouse button. When you have completed the definition of your ROI, click your right mouse button, then select the *Confirm Input* command in the context menu.
   - You will then once more see the *Colocalization* dialog box.
   - The ROI you have defined will now be shown in the preview image.
   - The scatterplot now only shows the intensity values for the pixels that are in the selected ROI.
10. If required, define further ROIs.



The illustration on the **left** shows a scatterplot. The image on the **right** shows the target channels and the colocalization channel. A ROI (**1**) has been defined on the image. Only the pixels within the defined ROI are represented in the scatterplot.

Viewing the results

11. Clear or select the check boxes next to the ROIs. To do this, click once in the box to the left of the ROI's name.

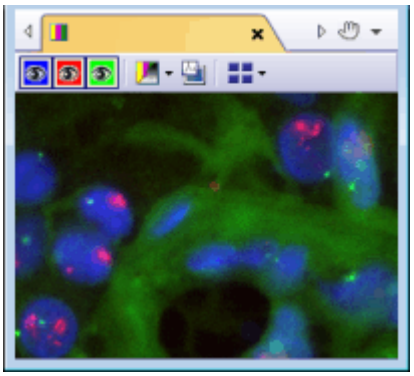


12. Pay attention to the displayed results in the preview, and in the *Results* group.
13. Click the *OK* button to finish the measurement of colocalization.
    - A workbook that contains the results of the colocalization measurement will be created. The workbook contains the measurement results for each ROI that has been defined.

10.4.3. Performing a measurement of colocalization on particular structures in the image

You can also measure the colocalization on image structures. Where images are concerned on which the image structures that are to be analyzed are numerous, and are spread over the whole image, this procedure is quicker than setting a lot of ROIs. If, for example, you want to measure the colocalization on image structures that have been stained with the (blue fluorescent) fluorochrome DAPI, select the blue channel. Then define the threshold values for this channel.

Example: On the image, the colocalization of the red and green pixels within the area marked in blue (cell nucleus) is to be measured. All other positions on the image are to be ignored.



The source image is a multi-channel image with three color channels.

1. Load the multi-channel image for which you want to carry out a colocalization measurement.
2. On the *Life Science Applications* toolbar, click the *Colocalization* button.
3. From the *Scatterplot > Channel 1* and *Channel 2* fields, select the two color channels on which you want to perform the measurement of colocalization. For this example, you can select the red and green channels.

Selecting the preview function

4. In the *Preview* group, click this button . Select the commands *Target channels and colocalization channel* and *Show target area* from the menu.
   - The active preview functions are identified with a check mark.

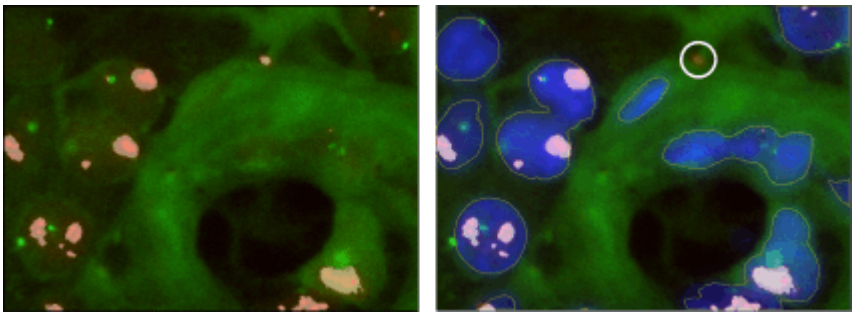
Defining thresholds for channel segmentation

5. In the *Target area* group, select the *Channel segmentation* entry from the *Area* list.
6. Click the button located next to the *Area* field, on its right-hand side.
   - A picklist with the different methods for setting threshold values, will open.
7. Select the *Automatic Threshold* method.
   - This method requires the user to make the smallest number of settings. Therefore, you should only use the other methods for setting threshold values, when the *Automatic Threshold* method doesn't lead to the result you wanted.

- The *Automatic Threshold* dialog box opens. Your software will carry out an automatic setting of threshold values. In the image window, you will now see the image structures that are detected by the automatic threshold settings.

8. Select the channel you want from the *Channel* group. For fluorescence images, select the *Dark* option in the *Background* group in the *Automatic Threshold* dialog box.
9. Close the *Automatic Threshold* dialog box with *OK*.
   - You will then once more see the *Colocalization* dialog box.
   - All available channels are displayed in the fields under the *Area* picklist.
10. Select the channel of the fluorochrome with which the image structure that is to be analyzed, has been stained. In the example, the *DAPI* channel has been selected.

   - The selected channel is now also shown in the preview.
   - The colocalized pixels that are shown fall exclusively within the image structures that are defined by the thresholds for the selected color channel.



The image on the **left** shows the preview image before the threshold values have been set. In the bright areas of the image, colocalization has been measured in the green and red color channels. The image on the **right** shows the preview image after the threshold values have been set. The blue color channel is now shown as well, because the measurement of colocalization is restricted to the blue image structures. The thin line around the blue structures shows which areas of the image have been selected by the setting of the threshold values. The white circle shows a position that is not on a blue cell. A measurement of colocalization was performed there before the threshold values were set.

11. If required, in the scatterplot, limit the intensity range that you want the measurement of colocalization to analyze.
12. Click the *OK* button to finish the measurement of colocalization.
    - A new image that contains the colocalization channel will be created.
    - At the same time, a workbook that contains the results of the colocalization measurement, will be displayed. The columns in the workbook contain the supplement [*Separation channel*].
    - In the source image, the segmentation image will additionally be displayed. The segmentation image shows the threshold value setting for each channel. Use the *Dimension Selector* tool window to show or hide the segmentation image.

13. If required, use the *File > Save As* menu command to save the new image and the workbook.
14. The multi-channel image will also have been changed when the channel segmentation was defined. If you do not want to keep these settings, do not save it.

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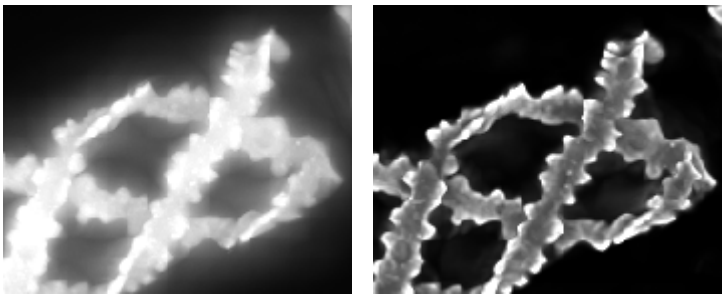
10.5. Deconvolution

The *Process > Deconvolution* submenu offers you deconvolution filters with which you can remove disturbing diffused light from an individual image or a multi-dimensional image. With a suitable parameter selection the image will become sharper and more clear.



Before using a deconvolution filter

The result of a deconvolution process largely depends upon whether certain parameters are known with which the image was acquired. These parameters include for example the objective's numerical aperture and refraction index. Before using a deconvolution filter on an image, use the *Process > Deconvolution > Verify Channel Parameters* command to check the relevant parameters for the image, changing them if necessary.

What is deconvolution?

In fluorescence and brightfield microscopy, diffused light from areas above or below the focal plane leads to over exposure, distortion and blurring. A suitable mathematical model to describe this problem is a convolution operation:

g(x) = f(x) * h(x) + n(x)
x: Point in XY space
g(x): observed image
f(x): ideal image
h(x): Point spread function
n(x): Noise function
*: Convolution

To be able to reconstruct the ideal image f(x) from the observed image g(x), you must know the noise function n(x) and the point spread function h(x). While an estimation of the noise function n(x) is highly possible, the point spread function h(x) depends

normally so strongly on the optical properties of the microscope and the sample, that an experimental determining of this function is not directly possible. For this reason, mathematical algorithms become necessary to even approximately determine the point spread function h(x) and to subsequently make the best possible reconstruction of the ideal image f(x) by means of deconvolution. A perfect, unambiguous, reconstruction is generally not possible, since information can be lost during a convolution.

The deconvolution filters

The individual deconvolution filters essentially differ in how the point spread and noise functions are determined, which are needed for the deconvolution of the image and the noise depression.
The more image data is used to calculate the point spread function, the more precise the result will be and the longer the calculation will take.

2D Deconvolution

The 2D deconvolution filter uses a theoretical point spread function, in which only the acquisition parameters are used but no image data.
You can apply the 2D deconvolution filter on all supported image types. However, the 2D deconvolution filter always affects only an individual frame.
As the amount of data processed is quite small, the 2D deconvolution filter is very quick. It makes the image appear much sharper but does not then permit any quantitative analysis of the image data.
The filter is especially well suited for TIRF images where the image information comes from a very narrow Z-range of the sample.

Nearest Neighbor Filter

The nearest neighbor filter employs a theoretical point spread function for the deconvolution, in the calculation of which, the data of the image under examination and of the two neighboring images of a Z-stack, are taken into consideration. The point spread function is applied to the neighboring images. The scaled sum of the neighboring images that have been processed in this way, is then deducted from the image under examination. With single images and simple time stacks the nearest neighbor filter works like a no neighbor filter. In this case, only the observed image's data are used in the calculation of the point spread function.

Wiener Filter

The Wiener filter approximates the point spread function by a linear function, with the mean square deviation being minimized. The actual filter is calculated from the linear inverse function. With Z-stacks the complete Z-stack's data are used in the calculation, with individual images only the observed image's data.

Constrained Iterative Filter

The *Constrained Iterative* filter does not make any presumptions about the point spread function, but rather extracts it directly from the Z-stack. This occurs iteratively. An estimated point spread function is used as a starting point. Then, an assumption is made as to which ideal image, via this point spread function, would have led to the observed image. Then an estimation has to be made as to which point spread function

caused the original image to be transformed into the observed image. This alternating assessment can be repeated as often as wished. Special mathematical processes are used to ensure that these iterations converge to reasonable values.

The *Constrained Iterative* filter promises the best results of all of the deconvolution filters, requires though, the most calculation time. Since the filter works iteratively on the complete Z-stack, a use on individual images or simple time stacks is not possible.

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10.6. Ratio Analysis

10.6.1. Overview - Ratio Analysis

What is a ratio analysis?
Certain multichannel fluorescence microscopy inspection modes allow you to monitor changes in ion concentration or pH value within cellular structures. Fluorescence dyes whose excitation characteristics depend on the concentration of ions are used for this.

The Fura-2 fluorescence dye, for example, shifts its excitation level from 340 nm to 380 nm when the calcium ion concentration decreases. At an excitation wavelength of 340nm, the intensity increases when the calcium concentration increases. At an excitation wavelength of 380nm it's the exact opposite. The higher the calcium concentration is, the less light is emitted.

The process flow of a ratio analysis on a multi-channel fluorescence image

1. Acquiring a multi-channel fluorescence image
The fluorescence dye is excited with two different wavelengths, one after the other. The multi-channel fluorescence image contains two color channels created with the same fluorescence dye, but at different excitation wavelengths.

The excitation wavelengths 340 nm and 380 nm are typically used with the Fura-2 fluorescence dye.

2. Carrying out background correction
A background correction is carried out on both color channels. You can make settings for the background correction in the *Ratio Analysis* dialog box.

3. Calculating a ratio image
One color channel is divided by the other on a pixel by pixel basis. The result is the ratio image, in which the intensity is proportional to the ion concentration.

When the Fura-2 fluorescence dye is used, the image acquired at the excitation wavelength of 340 nm is divided by the image acquired at the excitation wavelength of 380 nm:
Color channel (340 nm) / color channel (380 nm)

4. Viewing a ratio image
The result is a multi-layer image. One image layer is the source image and the other image layer is the ratio image. The ratio image displays the concentration of ions using pseudo colors. The pseudo color image is superimposed on the source image so that you can see the structures in your sample and the concentration of ions at the same time.

You can change the display of the resulting image in the image window, in order to view only the ratio image for example.

The process flow of a ratio analysis on a multi-channel time stack

1. Acquiring a multi-channel time stack

2. Carrying out background correction

3. Calculating an intensity profile

When the ratio analysis is carried out on a multi-channel time stack, you can compute the intensity profile in addition to the ratio image. The intensity profile displays the change in concentration of ions in a particular image segment. You determine the image segment by defining a ROI. You can define the ROI right here in the *Ratio Analysis* dialog box.

You can measure the intensity profile of several image segments at the same time.

4. Viewing a ratio image

The result is a multi-layer image. One image layer is the source image and the other image layer is the ratio image. The ratio image displays the concentration of calcium ions using pseudo colors. The pseudo color image is superimposed on the source image so that you can see the structures in your sample and the concentration of calcium ions at the same time.

You can change the display of the resulting image in the image window, in order to view only the ratio image for example.

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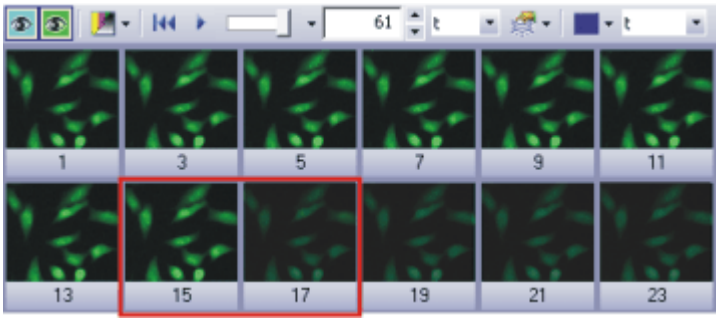
10.6.2. Measuring changes in the concentration of calcium ions in a time stack

Task: The Fura-2 fluorescence dye makes it possible to measure the concentration of free calcium ions because its excitation level shifts from 340 nm to 380 nm as the calcium ion concentration decreases. Use the ratio analysis to compute the ratio image over time and the intensity profile in two cells.



The image displays an overview of the frames in a multi-channel time stack that has 2 color channels. The sample has been dyed with the Fura-2 fluorescence dye. Between the frames that are framed in red in the illustration, the image intensity decreases visibly. The cause is a change in the calcium ion concentration.

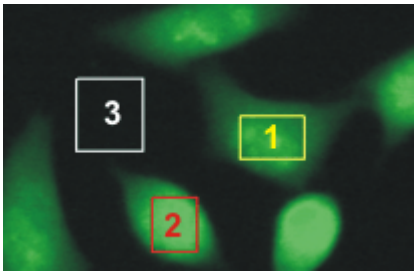
Preparing the analysis

1. Several example images were supplied together with your software. You can follow these step-by-step instructions using the Fura.tif example image. This example image is a multi-channel time stack image.
2. Use the *View > Toolbars > Life Science Application* command, to have the *Life Science Application* toolbar displayed. You can find the functions for defining ROIs and for Ratio Analysis on this toolbar.

Defining ROIs (Regions Of Interest)

3. Click the *New ROI - Polygon* button on the *Life Science Applications* toolbar.
4. Draw a rectangle inside a cell.
5. Define another ROI in a different cell.
6. Define another ROI in a dark image segment that has no fluorescing objects. This ROI will be used as a reference for the background correction.
7. Rename the ROIs you defined.

   To do this, open the *Measurement and ROI* tool window. In the *Measurement and ROI* tool window, double click on the first ROI's name. Enter a descriptive name for the ROI. Name the ROIs *Cell01*, *Cell02* and *Background*, for example.



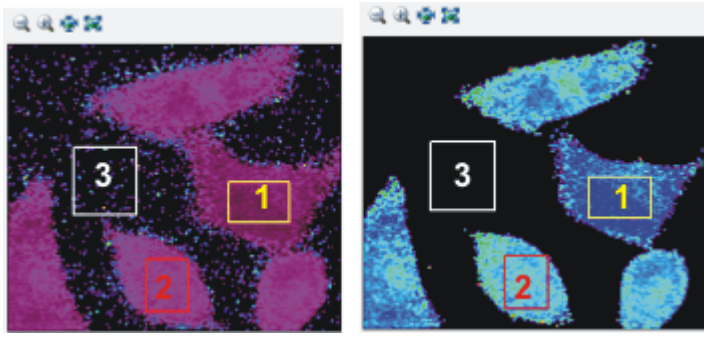
Three ROIs have been defined on the image. The red and yellow ROIs contain cells. The white ROI is on the background.

Carrying out a Ratio Analysis

8. Click the *Ratio Analysis* button located on the *Life Science Applications* toolbar.
   - The *Ratio Analysis* dialog box opens.
9. Make the settings for the background correction in the *Background* group. Select the *ROI* option. In both lists, select the reference ROI for the background correction.
10. In the *Ratio* group, select the parameters for the calculation of the ratio image. Select the *Fura340* color channel from the *Numerator* list and the *Fura380* color channel from the *Denominator* list.
    - The preview image in the *Ratio Analysis* dialog box displays the ratio image that has been computed for the time point that is currently displayed in the image window. The ratio image is the result of dividing the intensity of the Fura340 color channel by the intensity of the Fura380 color channel.
    - The ratio image is a gray-value image which automatically has a predefined pseudo color table applied to it in the preview window. High ratio values are displayed in red with this pseudo color table and low ratio values are displayed in magenta.

- The ratio image has single pixels with a high intensity in the background. This is image noise.

11. In the *Thresholds* fields, increase the value until the image noise disappears and only the cells remain visible.
12. In the *Scale* list, accept the value of 1000 that is given.

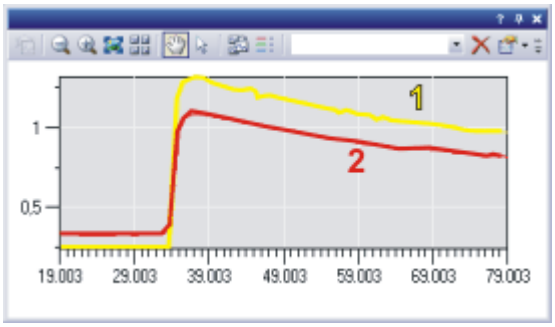


On the **left** you can see the preview image before the threshold values were set.
On the **right** you can see the preview image after the threshold values were set. The image background is now black. The colors in the preview image have changed because the image in the preview window is always displayed with the most possible contrast.

Viewing the results

13. In the *Output* group, select the *Image as new layer* and *Intensity Profile* check boxes.

    If you want to output the intensity profile as a sheet, select the *Export to workbook* check box.
14. Select the ROIs that you defined on the cells. The ROIs you have selected are highlighted in the dialog box.
15. Close the *Ratio Analysis* dialog box with *OK*.
    - If it wasn't already displayed, the *Intensity Profile* tool window is displayed automatically now. The tool window contains two intensity profiles, one for each ROI you defined.



The intensity profiles show how the ratio value in both ROIs (**1**) and (**2**) changes over time. The colors of the intensity profiles correspond to the colors of ROIs they describe.

- The source image is now a multi-layer image and displays the concentration of calcium ions in addition to the image information.

16. Use the *File > Save As* command to save the resulting image. Save the resulting image in the TIF or VSI file format.

10.6.3. Setting the display of the ratio image

1. Carry out a ratio analysis.
   - The image resulting from a ratio analysis is a multi-layer image. One image layer is the source image and the other image layer is the ratio image. There are several ways of displaying the resulting image on the monitor.
2. Use the *View > Tool Windows > Layers* command to make the *Layers* tool window appear. You have access to the individual image layers in the *Layers* tool window.
3. Select the *View > Tool Windows > Adjust Display* command to make the *Adjust Display* tool window appear. In the *Adjust Display* tool window, you can specify how an image is displayed on the monitor.
4. Activate the image resulting from the ratio analysis in the document group.

Browsing the time stack

1. Take a look at the peak in the intensity profile in this time stack.
   To do this, use the navigation bar at the top of the image window.
   
2. If the info stamp isn't displayed in the image window, use the *View > Info Stamp* command to display it.
   - The info stamp should display the time for each frame.
   - If this time is not on display, select the *Tools > Options* command.

     In the tree view, select the *Info Stamp > Properties* entry. In the *Available properties* list, select the *Image > t* check box.

     Close the dialog box with *OK*.

Viewing the ratio image and the source image separately

You can view only the ratio image or only the source image in the image window whenever you want.

1. In the *Layers* tool window, click once on the source image to select this image layer. The name of the image layer in the tool window corresponds to the name of the image.
2. Click once on the eye icon next to the ratio image.
   - The ratio image is now not displayed in the image window. You see only the source image.
3. In the *Layers* tool window, click once on the ratio image to select this image layer.
   - When you select an image layer in the *Layers* tool window, this image layer is automatically displayed.
4. Click once on the eye icon next to the source image.
   - The source image is now not displayed in the image window. Now you see only the ratio image.

The illustration shows the *Layers* tool window with the image resulting from a ratio analysis.
On the left, the ratio image (**1**) is not displayed. On the right, the source image (**2**) is not displayed.

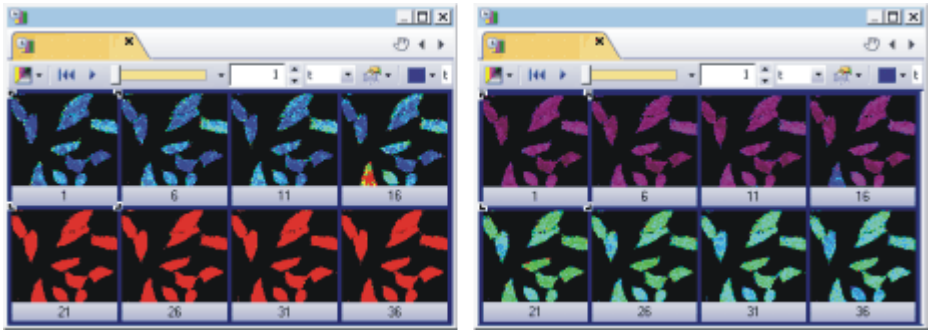
Optimizing the display of the ratio image

1. Display only the ratio image in the image window.
2. Use the *Adjust Display* tool window to optimize the display of the ratio image.
3. Select the *Auto Contrast* option.

   This makes sure that the ratio image is displayed in the image window with the most possible contrast. With this setting, all the colors in the pseudo color table you are using are applied to the ratio image.

   The *Histogram of all frames* check box decides whether only the frame in the time stack that is currently on display will have its contrast optimized or whether the contrast will be optimized across all the frames in the time stack.
4. Select the *Histogram of all frames* check box.

   Your software now takes the smallest and largest values in all the frames and assigns the colors black and red to these values.
5. Click the *Apply* button to make the changed settings visible in the image window.



The illustration shows the same ratio image where different settings have been made in the *Adjust Display* tool window.
On the **left**, the contrast has been optimized for the first frame. The *Histogram of all frames* check box is not checked. Because the values in the ratio image increase over time, the color shifts towards red.
On the **right**, the contrast was optimized across all frames. The *Histogram of all frames* check box was selected. With this setting, differences in the ratio image can be seen in all frames.

Viewing the ratio image and the source image at the same time

1. Display all the image layers in the image window. In the *Layers* tool window, you can see an eye icon next to each image layer.
2. Select the ratio image in the *Layers* tool window, and click your right mouse button to open a context menu.

3. Select the *Mode > Intensity Modulation* command from the context menu. If this mode is already set, keep it.
   - The colors in the ratio image remain unchanged and their color value reflects their ratio value.
   - The intensity of the ratio image is adjusted to the intensity of the source image. Where there is a low intensity in the source image, the ratio image is also dark.

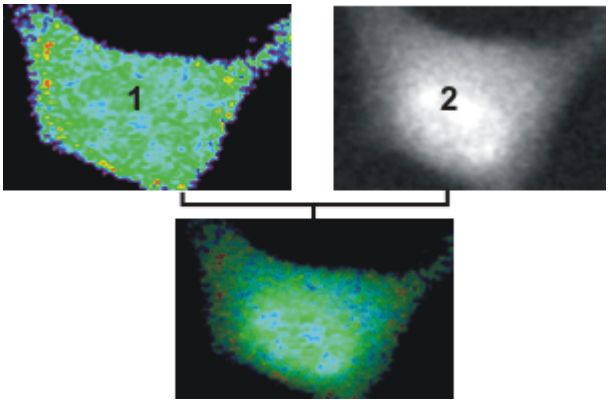


Image (**1**) is only the ratio image. The colors are all equally bright. Image (**2**) is the source image. Your software hasn't applied color mapping to it.
In the image at the bottom, the intensity in the ratio image corresponds to the intensity in the source image. The intensity decreases noticeably towards the edges of the cell.

Displaying the color bar

1. Use the *View > Color Bar* command or the [Shift + F6] keyboard shortcut to show or hide a color bar with a default pseudo color table in the image window.
   - The color bar shows the distribution of the ratio values in the ratio image. High ratio values are displayed in red and low ratio values are displayed in magenta.

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10.7. FRAP

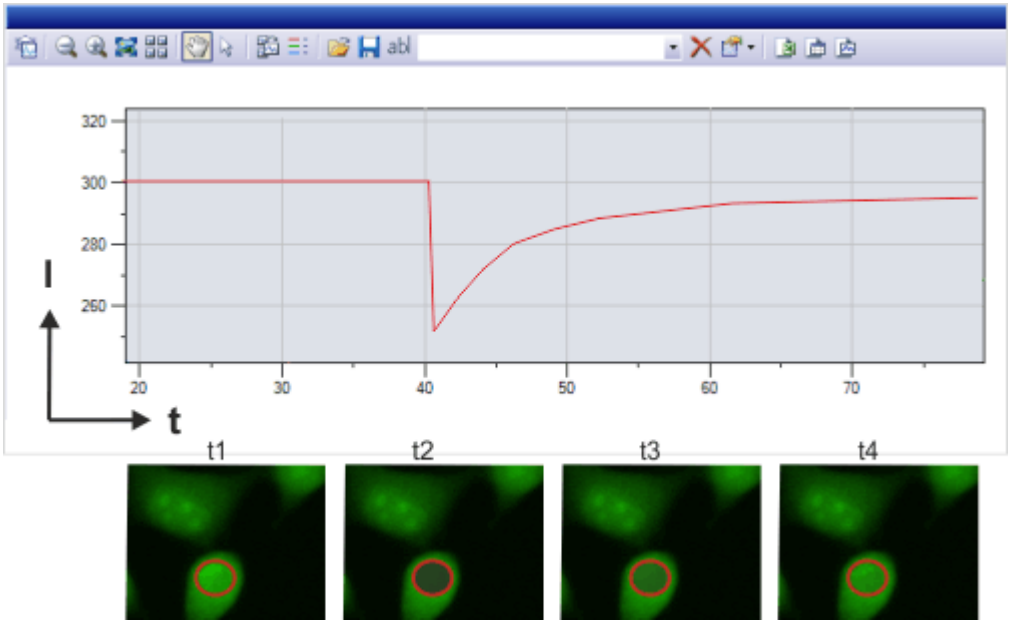
10.7.1. Overview - FRAP

Your software enables you to perform FRAP experiments. A FRAP experiment involves acquiring a time stack. During the acquisition, you illuminate one or more image segments with a laser. You can then perform a FRAP analysis on the time stack you acquired.

What is FRAP?

FRAP (Fluorescence Recovery after Photobleaching) is an inspection mode in fluorescence microscopy. It examines molecules that have been stained with fluorescence dyes. During a FRAP experiment, the intensity profile of particular positions on the sample is measured over time. During the experiment, these positions on the sample are illuminated with a laser. The laser destroys the fluorescing molecules. This bleaches the sample locally (photobleaching). The recovery of the

intensity of the fluorescent light in the bleached positions on the sample is examined. The recovery could be caused by the diffusion of molecules from neighboring areas of the sample, or the generation of new proteins.



The images at the bottom show several frames in a time stack on which a circular ROI (Region of Interest) has been defined. The chart above shows the intensity profile within the ROI.
At time point t2 the area within the red ROI is illuminated with a laser. The intensity within the ROI drops suddenly. As the time stack progresses, the intensity within the ROI increases again. This can happen due to diffusion processes, for example.

Note: The Olympus-FRAP-System also enables other experiments which require a laser to be directed precisely on the sample.

Prerequisites for FRAP

Prerequisite: The FRAP functions are only available if you purchased the *Photo Manipulation* solution together with your software.

Hardware requirements

For the FRAP method you require a fluorescence microscope with special hardware:

- one or more FRAP lasers for bleaching the sample. If you want to use more than one FRAP laser, you need a laser combiner.
- a FRAP laser scan system. The FRAP laser scan system has a control box that controls the scan system and the FRAP laser shutter.
  This FRAP laser scan system can be mounted in the light path of an Olympus IX3 microscope, the IX73 P2F for example. The FRAP laser scan system allows the FRAP laser to be directed to specific positions on the sample.
- an Olympus RTC (real time controller) Your software uses the RTC to control the FRAP laser, the laser scan system and the image acquisition.

Software requirements

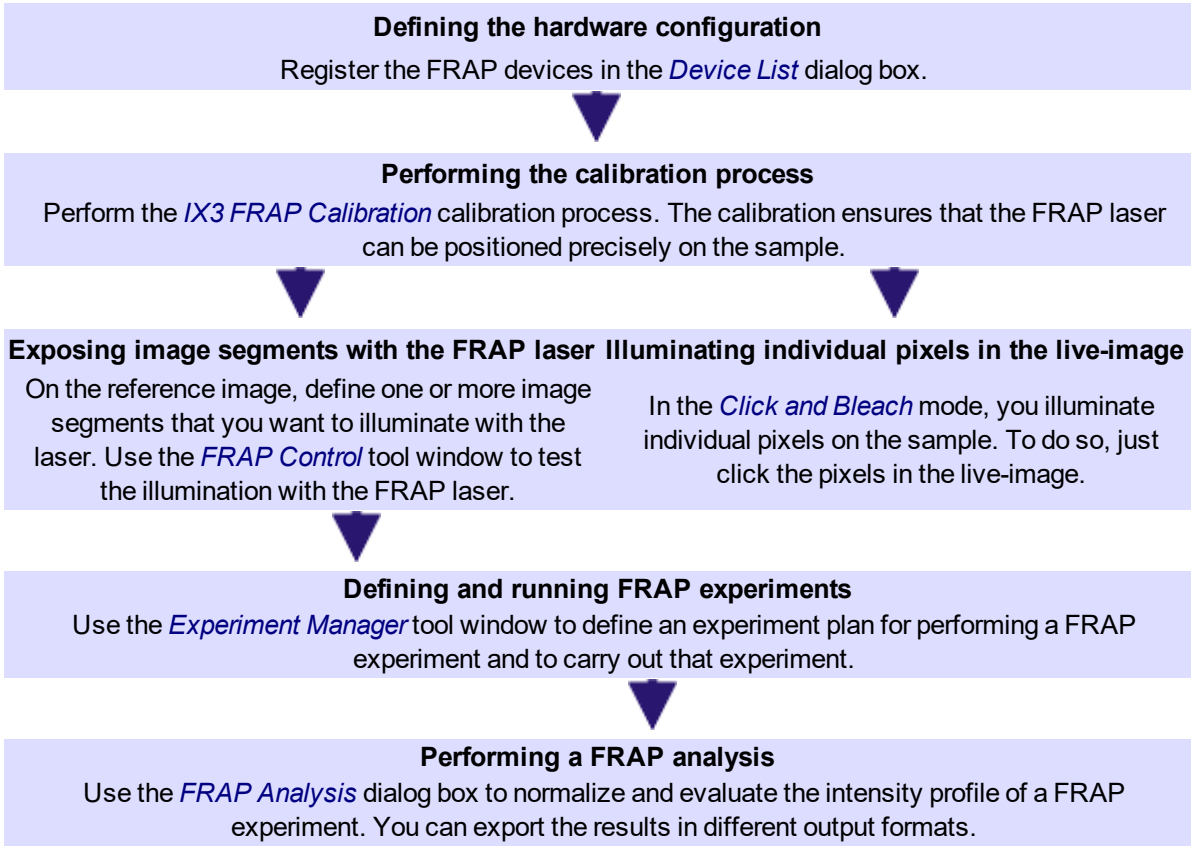
All FRAP devices have to be selected during the installation of the software.

When you want to acquire multi-channel fluorescence images, it makes sense to define observation methods for your color channels before you define the experiment. Only when you've defined an observation method can you assign a fluorescence color to the individual color channels when acquiring fluorescence images, for example.

You can find step-by-step instructions on the definition of observation methods for fluorescence acquisition here.

The general course of a FRAP experiment

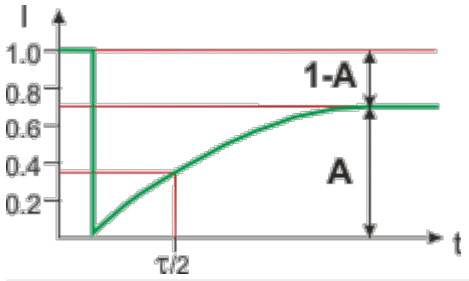
The following process flow chart shows the steps required to perform a FRAP experiment.



FRAP analysis

The FRAP analysis normalizes and evaluates a FRAP experiment's intensity profile.

After the bleaching of the sample with the FRAP laser, the fluorescence intensity recovers. But the intensity is now lower than it was before the bleaching. This can be the result of irreversible damage to the molecules caused by the FRAP laser. Values that are characteristic for this process can be ascertained from the intensity profile.



The illustration shows the intensity profile and the characteristic values.
Immobile Fraction (**1-A**), Mobile Fraction (**A**), and **τ/2**.

The value *A* is the mobile fraction and is the maximum relative intensity value that is regained after the bleaching of the sample.

The value *1-A* is the immobile fraction and is the difference between the fluorescence intensity before the bleaching and the maximum intensity value after the bleaching.

The *τ/2* value is the interval after which the fluorescence intensity rises to half of the maximum value after bleaching.

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10.7.2. Defining the hardware configuration

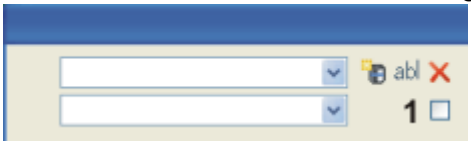
Preconditions

- For a FRAP experiment you require one or more FRAP lasers, the FRAP laser scan system, an Olympus RTC (real time controller) and the camera. All devices have to be selected during the installation of the software.
- The software and all controllable devices have been installed and connected to the PC and to the RTC.
- The camera driver is installed in MS-Windows.
- The network connection to the RTC has been configured.
- All of the devices have been turned on.

Registering devices

Use the *Device List* dialog box to register all of the FRAP devices with your software.

1. Use the *Acquire > Devices > Device List* command.
2. In the *Device List* dialog box, select the *RTC* check box. You will find the check box (**1**) to the right, next to the *Microscope Frame* list. The check box is only shown if the RTC was selected during the installation of the software.



Selecting a camera

3. Activate the *Camera* tab.
4. Select your camera and its port from one of the *Camera* lists.

Selecting a FRAP laser scan system

5. Activate the *Microscope* tab.
   - With IX3 series microscopes you have the option of mounting a FRAP laser scan system in the first or the second deck. Select the *IX3 FRAP* entry from the *Deck 1 (upper)* or the *Deck 2 (lower)* list.

Selecting a FRAP laser

6. Activate the *Lasers/LEDs* tab.
7. In the *Device* list, select the *FRAP Laser* entry. If you are using more that one FRAP laser, select the *FRAP Combiner* entry.
8. From the *Type* list, select the laser(s) that you want to use for your FRAP experiments. You can only use lasers that are connected to the RTC. All of these lasers begin with *RTC Laser*.
   - The *Shutter* and *Intensity* check boxes are automatically selected for the FRAP lasers.

Closing the device list

9. Click the *OK* button to confirm the hardware configuration entered.
   - The *Device List* dialog box will be closed.
   - The changed hardware configuration is automatically saved.

10.7.3. Performing the calibration process

Perform the *IX3 FRAP Calibration* calibration process for each FRAP laser. The calibration ensures that the FRAP laser can be positioned precisely on the sample.

Prerequisite. For the FRAP calibration process you require a special calibration standard that has a homogeneously fluorescing area. Olympus delivers one of these calibration standards together with the *Photo Manipulation* software solution.

Preparing the software user interface

1. Select an observation method that allows you to see the FRAP laser in the live-image.
2. Select an objective that you want to use later for FRAP experiments.
   - If you are using a magnification changer with your microscope, select the combination of objective and magnification changer setting here.
3. Use the *View > Tool Windows > Camera Control* command to make the *Camera Control* tool window appear. You can switch on the live-image and optimize the exposure time here.
4. Use the *View > Tool Windows > FRAP Control* command to make the *FRAP Control* tool window appear. You need the tool window to control the FRAP laser.
5. Hide information in the image window that could cover parts of the image, the scale bar and the info stamp for example. To do this, use the relevant commands in the *View* menu.
6. Use the *View > Tool Windows > Adjust Display* command to make the *Adjust Display* tool window appear.

In the *Adjust Display* tool window, select the *Auto Contrast* option.
In the *Auto Contrast > Right* field, enter the value 0.

- This setting prevents the bright laser spot in the image from always being overexposed.

Preparing the live-image

7. Place the FRAP calibration standard on the stage.
8. Switch to the live-image.

   If the laser spot is hard to see, change the focus or the intensity of the FRAP laser.

Starting a calibration process

9. Use the *Acquire > Calibrations* command.

   Select the *IX3 FRAP Calibration* calibration process in the *Calibrations* dialog box.

   Click the *Calibrate* button to start the software wizard.

   - Your software will automatically switch to the live mode.
   - In the *Calibration* dialog box, all of the objectives that are currently entered in device settings are listed. If you are using a magnification changer with your microscope, its settings are also shown.

10. Select the check boxes next to the objectives that you want to use for the FRAP experiments.
    - You have the choice between a manual or an automatic calibration. The manual calibration is described in these step-by-step instructions.
11. Select the *Manual calibration* check box. Use the number suggested in the *Calibration points* field.
12. Click the *Next >* button.
    - If you are working with more than one FRAP laser: All of the FRAP lasers are listed in the calibration dialog box.
13. Select the check boxes next to the FRAP lasers that you want to use for the FRAP experiments.
14. Click the *Next >* button.
    - The FRAP laser should now appear as a bright spot in the live-image.

    - If the FRAP laser isn't visible in the live-image, click the *Center Laser Spot* button. You can find this button in the *FRAP Control* tool window's toolbar.
    - If the FRAP laser still isn't visible in the live-image, it could be that your camera's field of view is too big. If this is the case, cancel the calibration process. Reduce the size of your camera's image area in the *Camera Control* tool window. Select a central area.
    - The calibration takes place in two steps. First a coordinate system is defined with three points. A grid is specified for the fine calibration that follows, made up of 4, 16, 36 or 64 points.
15. Click the *Start calibration* button to start the rough calibration.
    - A cross hair now appears in the live-image.

16. Click once in the center of the laser spot in the live-image.

    If the laser spot is very small, rotate the mouse wheel to enlarge the live-image in the image window.

    - Your laser now links the current XY position with the current setting of the FRAP laser scan system.
    - The laser is automatically directed to the second of a total of three points.
    - If the laser spot is now no longer visible in the image, click the *Move laser spot* button.
17. Click on the second laser spot and then on the third.
    - The rough calibration is now complete.
18. The fine calibration begins automatically. To do this, your software positions the FRAP laser on all of the points in the grid sequentially. In the live-image, click on the center of the laser spot each time.
    - When the calibration is complete, you automatically go back to the *Calibration* dialog box.
19. Close the *Calibrations* dialog box.
    - You can use the *FRAP Control* tool window to control the FRAP laser scan system and to illuminate particular areas on your sample.

10.7.4. Exposing image segments with a FRAP laser

Task: On the reference image, define one or more image segments that you want to illuminate with the laser. Use the *FRAP Control* tool window to test the illumination with the FRAP laser.

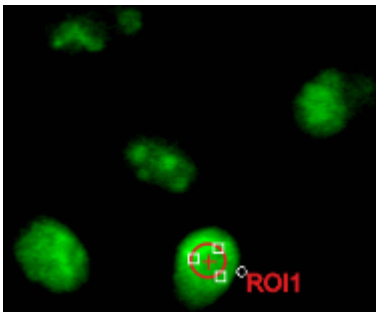
Acquiring reference image

1. Acquire an image of the sample that you want to bleach with the FRAP laser.
   - The acquired image could be called *Image_01*, for example, and is used as a reference image.

Defining ROIs (Region Of Interest)

2. Use the *View > Toolbars > Life Science Application* command, to have the *Life Science Application* toolbar displayed. You can find the functions for defining ROIs and for measuring the intensity profile on this toolbar.

3. Click the *New ROI - 3 Point Circle* button on the *Life Science Applications* toolbar.
4. Left click to define a circular ROI on the image.
5. If necessary, define further ROIs on the image.
   - You can now illuminate the area within the ROI with the FRAP laser to bleach the fluorochrome.



A circular ROI has been defined on the reference image.

Making settings for illumination with the FRAP laser

6. If you are working with more than one FRAP laser: In the *FRAP control* tool window, select the FRAP laser with which you want to illuminate the sample. Specify the intensity of the laser.
7. In the *FRAP Control* tool window, click the *FRAP* button.

   - At the bottom of the *FRAP Control* dialog box, the *Bleaching* group now appears.
8. Make the following settings in the *Bleaching* group:

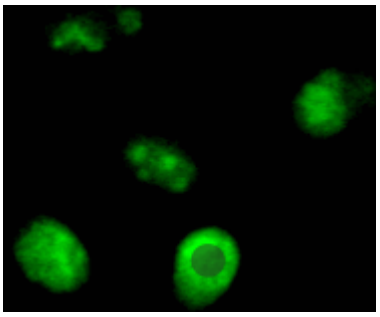
   Select the name of the reference image you just acquired from the *Reference Image* list, *Image_01* for example.

   Select the *Select all* check box to take into account all of the ROIs on the selected reference image.

   Select the *ROI Area* entry from the *Bleach Mode* list.
9. Select the *Continuous* check box. You can find the check box directly under the *Start* button, in the *FRAP Control* tool window.
10. Click the *Live* button in the *FRAP Control* tool window's toolbar to observe the bleaching of the sample in the live-image.

11. Click the *Start* button.
    - Before the actual illumination starts, all the necessary data is transferred to the connected FRAP devices. The progress of this process is displayed by the green bar in the *Transfer Status* field. When all of the data has been transferred and the whole bar is green, the illumination of the sample starts.
    - The FRAP laser now continuously scans the area within the ROI.

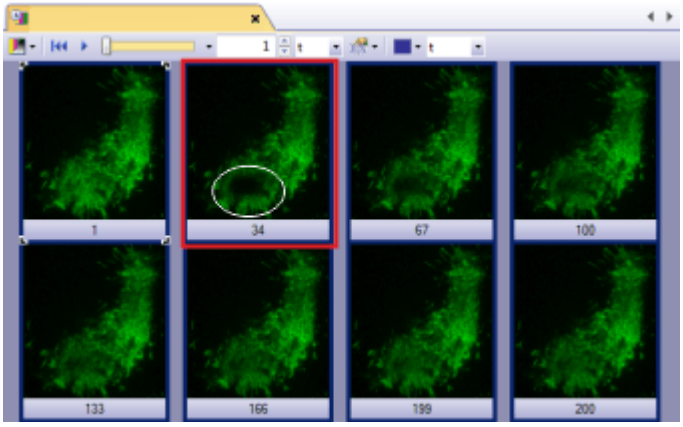
11. Click the *Stop* button to abort the illumination of the sample with the FRAP laser.
12. Acquire an image of the bleached position on the sample. To do this, you can use the *Acquire > Snapshot* command.



After the area of the sample within the defined ROI has been illuminated with the FRAP laser, it is bleached. The area lights up noticeably less in the fluorescence image.

10.7.5. Performing a FRAP analysis

Task: You have performed a FRAP experiment and have acquired a multi-channel time stack. Create an intensity profile from the position on the sample that is illuminated by the FRAP laser. Then evaluate this intensity profile.



The image displays an overview of the frames in a multi-channel time stack. Before the frame outlined in red in the illustration was acquired, a FRAP laser illuminated the area circled in white on the sample. The intensity of the fluorescence first decreases and then increases again.

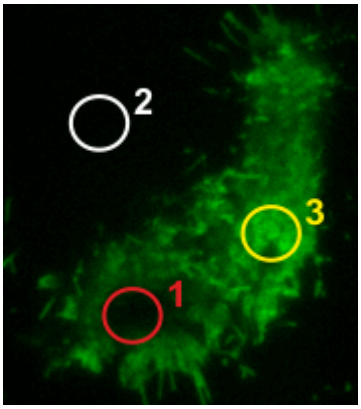
1. Load the multi-channel time stack that you acquired with the FRAP experiment.
2. Use the *View > Toolbars > Life Science Application* command, to have the *Life Science Application* toolbar displayed. You can find the functions for defining ROIs and for Ratio Analysis on this toolbar.

Defining ROIs (Regions Of Interest)

3. In the image window, use the navigation bar to display a frame in which the area of the sample that was illuminated by the FRAP laser is easy to see.
4. Click the *New ROI - 3 Point Circle* button on the *Life Science Applications* toolbar.
5. With three clicks of the mouse, define the ROI on the area of the sample that was illuminated by the FRAP laser.
   - The first ROI that you define on the image is automatically called *ROI1*.
6. Define another ROI (*ROI 2*) in a dark image segment that has no fluorescing objects. This ROI will be used as a reference for the background correction.

7. Define another ROI (*ROI 3*) on a position on the sample that is fluorescing but is not illuminated by the FRAP laser.
8. Rename the ROIs you defined.

   To do this, open the *Measurement and ROI* tool window. In the *Measurement and ROI* tool window, double click on the first ROI's name. Enter a descriptive name for the ROI. Name the ROIs *FRAP*, *Photobleaching*, and *Background* for example.

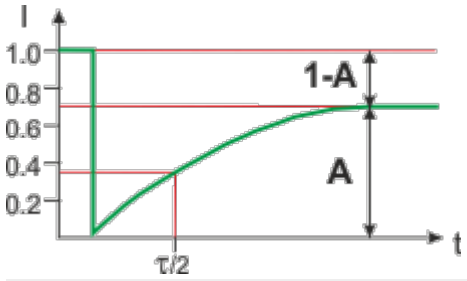


Three ROIs have been defined on the image. **ROI1** is in the area of the sample that was illuminated by the FRAP laser. **ROI2** is on the background. **ROI3** is on a fluorescing sample area that wasn't illuminated.

Performing a FRAP analysis

9. Click the *FRAP Analysis* button located on the *Life Science Applications* toolbar.
   - The *FRAP Analysis* dialog box opens.
   - In the *Stimulated ROI* list, all of the ROIs that have been defined on the current image are listed.
10. Click the *Default* button, to return all settings for the display of the intensity profiles to their default.
11. Select the color channel on which you want the FRAP analysis to be performed from the *Channel* list. The first fluorescence channel of the active image is selected by default.
12. In the *Stimulated ROI* list, select the check box next to a ROI that contains a bleached position on the sample. In this example, select the *FRAP* ROI.
    - The intensity profile within this ROI is displayed in the *Raw data* chart at the top left of the dialog box.
13. Make the settings for the background correction in the *Background* group.

    Select the *ROI* option. Select the ROI for the background from the list.
14. From the *Photo bleaching correction* list, select the ROI that is on an unilluminated fluorescing area of the sample
    - The normalized and corrected intensity profile is displayed in the *Normalized data* chart at the top right (the green curve).

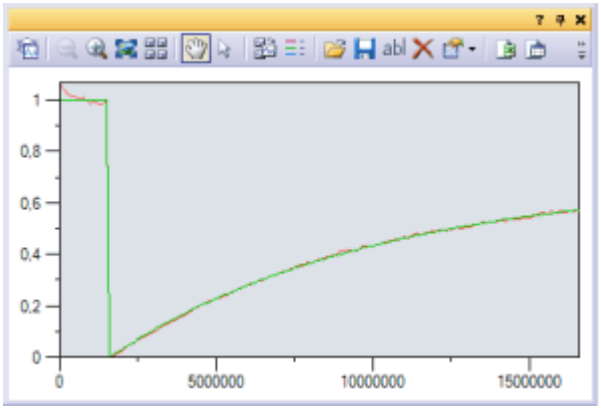


The illustration shows the intensity profile and the characteristic values.
Immobile Fraction (**1-A**), Mobile Fraction (**A**), and **τ/2**.

Viewing the results

15. Click the *Options* button.
    - The *Options > Measure > FRAP* dialog box opens.
16. Select the *Intensity Profile* check box in the *Output Options* group.
    If you want to output the intensity profile as a sheet, select the *Workbook* check box.
17. Close the *Options* dialog box with *OK*.
18. In the *FRAP Analysis* dialog box, click the *Execute* button.
    - If it wasn't already displayed, the *Intensity Profile* tool window is displayed automatically now. The tool window contains two intensity profiles. The green curve is the normalized and corrected intensity profile from which the results were calculated. The red curve is the normalized raw data.

16. You can save the intensity profiles. To do this, click the *Save intensity profile* button in the *Intensity Profile* tool window's toolbar.



The *Intensity Profile* tool window showing the results of a FRAP analysis.

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10.8. FRET

10.8.1. Overview - FRET Analysis

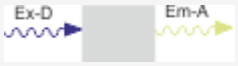
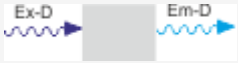
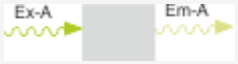
What is FRET?

FRET stands for Förster Resonance Energy Transfer. It measures the non-radiative energy transfer between 2 different fluorochromes, from the excited donor to the acceptor. The results are either the FRET index or the FRET efficiency. These two values tell you something about the interactions between proteins as well as distances. A typical example of fluorescence colors are CFP being used as a donor and YFP as an acceptor.

Prerequisites for performing a FRET analysis with your software

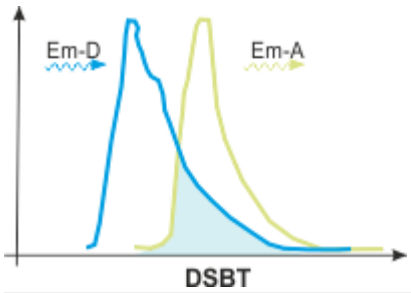
Your software makes the *Measure > FRET Correction* and *Measure > FRET Analysis* commands available. Both commands require fluorescence images suitable for FRET correction and FRET analysis to be available.

For the acquisition of fluorescence images for FRET correction and FRET analysis, three different combinations of excitation and emission filters have to be set in the fluorescence microscope:

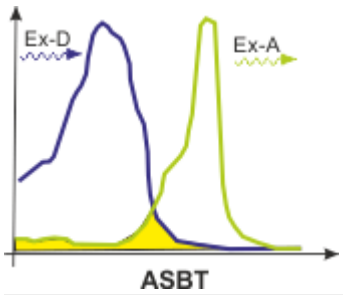
Ffret		The sample is illuminated with the donor's excitation light (**Ex-D**) and the acceptor's emission light (**Em-A**) is observed.
Fdon		The sample is illuminated with the donor's excitation light (**Ex-D**) and the donor's emission light (**Em-D**) is observed.
Facc		The sample is illuminated with the acceptor's excitation light (**Ex-A**) and the donor's emission light (**Em-D**) is observed.

FRET Correction

Both the spectral bleed through (SBT) in the acceptor channel (DSBT) from the donor emission and also the excitation of the acceptor molecule by the donor excitation (ASBT) are superimposed on the FRET signal.

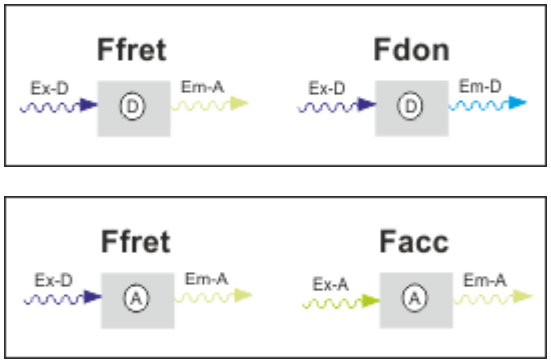


The illustration shows the donor's and the acceptor's emission spectra. The spectra overlap. When a sample that contains the donor is observed in the acceptor's emission light, as is the case with FRET experiments, a portion of the observed light intensity comes from the emission of the donor. This emission light is not created by the FRET effect.



The illustration shows the donor's and the acceptor's excitation spectra. The spectra overlap. When a sample that contains the acceptor is illuminated with the donor's excitation light, this light also with a certain probability excites the acceptor to light up.

Use the *Measure > FRET Correction* command to specify correction factors for this effect using reference images. To specify the DSBT=Ffret/Fdon and ASBT=Ffret/Facc factors, your require two images of a sample that only contains the donor and two images of a different sample that only contains the acceptor.



You require two samples to specify the DSBT and ASBT FRET correction factors. The first should only contain the donor and the second should only contain the acceptor. The illustration shows the required acquisition conditions.

FRET Analysis

For a FRET analysis, you require fluorescence images of a sample that has been dyed with two suitable fluorescence colors. In a FRET experiment the dyes take on the function of the donor and the acceptor. One of the dyes, the donor, is excited. The fluorescence intensity of the other dye, the acceptor's, is observed.

The *FRET Analysis* dialog box offers different methods of computing the *Ffret*, *Fdon* and *Facc* source images in relation to each other. These computation methods are taken from the following publications:

- Xia, Liu. 2001. Reliable and Global Measurement of FRET Using Fluorescence Microscopes. Biophys. J. (81), 2395-2402
- M.Elangovan, H.Wallrabe, Y.Chen, R.N.Day, M.Barroso and A.Periasamy. 2003. Characterization of one- and two-photon excitation fluorescence resonance energy transfer microscopy. Methods 29 (2003) 58-73
- Gordon et al. 1998. Quantitative Fluorescence Resonance Energy Transfer Measurements Using Fluorescence Microscopy. Biophys. J. (74), 2702-2713
- Youvan, D. C., W. J. Coleman, C. M. Silva, J. Petersen, E. J. Bylina, and M.M. Yang. 1997. Fluorescence imaging micro-spectrophotometer. Biotechnology et alia. 1:1–16

The FRET analysis results in a computed image in which the intensity corresponds either to the FRET index or to the FRET efficiency, depending on which computation method was selected. The resulting image is added to the FRET image in an additional image layer. The FRET image is the source image that was illuminated with the donor's excitation light (Ex-D) and observed in the acceptor's emission light (Em-A).

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10.8.2. Specifying correction factors for a FRET analysis

Task: You want to perform a FRET analysis with the CFP and YFP fluorescence dyes. CFP is the donor and YFP is the acceptor.
Specify the DSBT and ASBT correction factors. DSBT is the spectral bleed through in the acceptor channel from the donor emission. ASBT is the excitation of the acceptor molecule by the donor excitation.

Prerequisite: Reference samples are available that each contains only one of the fluorescence dyes. In this example, one sample that only contains the CFP dye and another sample that only contains the YFP dye are required.

Acquiring reference images

1. Acquire two fluorescence images of the donor sample. To do so, use the *Ffret* and *Fdon* acquisition conditions. Use the same exposure time for both fluorescence images.

Ffret		The sample is illuminated with the donor's excitation light (**Ex-D**) and the acceptor's emission light (**Em-A**) is observed.
Fdon		The sample is illuminated with the donor's excitation light (**Ex-D**) and the donor's emission light (**Em-D**) is observed.

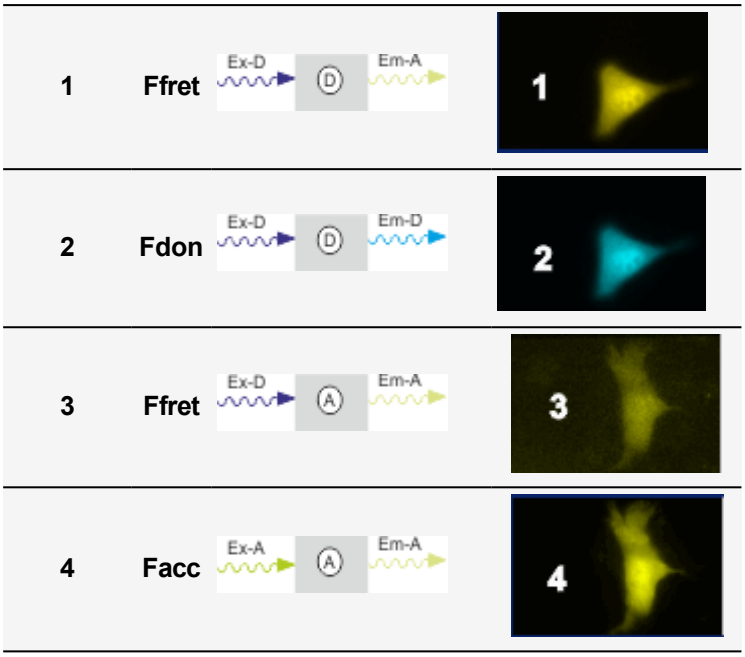
2. Acquire two fluorescence images of the acceptor sample. To do so, use the *Ffret* and *Facc* acquisition conditions. Use the same exposure time for both fluorescence images.

Ffret		The sample is illuminated with the donor's excitation light (**Ex-D**) and the acceptor's emission light (**Em-A**) is observed.
Facc		The sample is illuminated with the acceptor's excitation light (**Ex-A**) and the acceptor's emission light (**Em-A**) is observed.

Note: Depending on the microscope configuration, you can acquire individual fluorescence images or multi-channel fluorescence images. However, the reference images must be of the *multi-channel image* image type.

Loading reference images

3. Load the reference images in your software's document group. The following table shows example images.

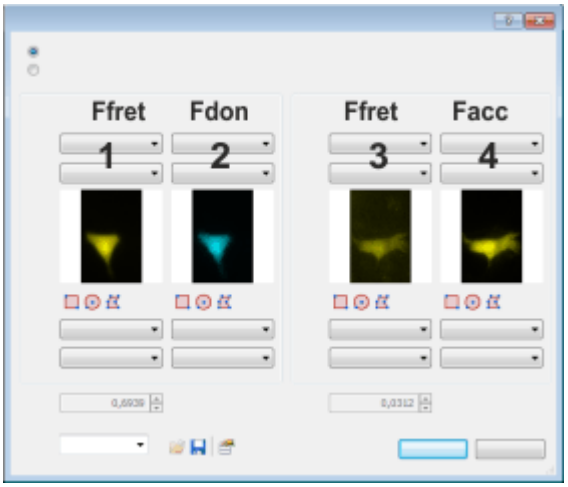


4. Take a look at the reference images.
   - The images of the donor (**1**, **2**) don't only show emission in the donor's blue channel (**2**), but also in the acceptor's yellow channel (**1**).
   - The image of the acceptor, that is excited in blue (**3**) also shows emission in the acceptor's yellow channel.

Correcting spectral bleed through

5. Click the *FRET Correction* button located on the *Life Science Applications* toolbar.
   - The *FRET Correction* dialog box opens.
6. Select the *Calibrate* option.
   - The *Input Donor sample* and *Input Acceptor sample* groups become active.
   - All of the multi-channel images that are currently loaded in your software are listed in the *Image* list.
7. Select reference images **1**-**4**, described above, in the *Image* and *Channel* lists.

   If you are using multi-channel images as reference images, you have to select the image and the color channel.



Select the appropriate reference images.

Carrying out background correction

8. Select two ROIs on each image for the background correction.

   You can do this by clicking the *Create rectangle ROI* button under the image (**1**).

   - Your software closes the dialog box and automatically activates the corresponding image in the image window.
9. Define a ROI in a dark image segment that has no fluorescing objects. This ROI will be used as a reference for the background correction.

10. Right click and select the *Confirm Input* command to return to the *FRET Correction* dialog box.
11. Define another ROI in an image segment that does have fluorescing intensity.
12. Select the appropriate ROIs from the *ROI signal* and *ROI background* lists.
    - The correction factors that were determined are now displayed in the *DSBT a (Ffret/Fdon)* and *ASBT b (Ffret/Facc)* fields.

The DSBT and ASBT correction factors are displayed at the bottom of the *FRET Correction* dialog box.

Saving correction factors

13. Enter, for example, *cfp-yfp* in the *Name* field and click the *Save* button.
    - You can now use these correction factors for a FRET analysis any time.
14. Close the *FRET Correction* dialog box.

10.8.3. Performing a FRET analysis

Task: You want to perform a FRET analysis with the CFP and YFP fluorescence dyes. CFP is the donor and YFP is the acceptor.
Analyze the FRET images using the Gordon (1998) computation method.

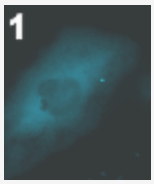
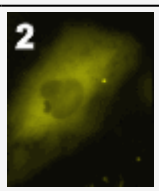
Acquiring FRET images

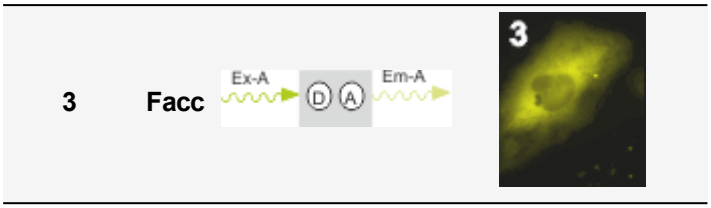
1. Acquire three fluorescence images of the FRET sample that contain both the donor and the acceptor fluorescence dye. To do so, use the *Fdon*, *Ffret* and *Facc* acquisition conditions. Use the same camera settings for all of the FRET images, especially the same exposure times.

Fdon		The sample is illuminated with the donor's excitation light (**Ex-D**) and the donor's emission light (**Em-D**) is observed.
Ffret		The sample is illuminated with the donor's excitation light (**Ex-D**) and the acceptor's emission light (**Em-A**) is observed.
Facc		The sample is illuminated with the acceptor's excitation light (**Ex-A**) and the acceptor's emission light (**Em-A**) is observed.

Loading FRET Images

2. Load the FRET images in your software's document group. The following table shows example images.

1	**Fdon**		
2	**Ffret**		



3. View the FRET images.
   - The images with donor excitation (**1**, **2**) show the emission of the donor in the blue channel (**1**), and in the yellow channel (**2**) they show the emission of the acceptor due to the FRET effect. The donor's emission light and the acceptor's fluorescence light that has been excited by the donor's excitation light, also contribute to the intensity that you observe in the FRET channel (**2**). These portions are subtracted from the observed intensity in the FRET channel.
   - The image with the acceptor excitation (**3**) shows the emission of the acceptor in the yellow channel.

Defining ROIs for background correction

4. Define a ROI on each image in a dark image segment that has no fluorescing objects.
   You can click the *New ROI - Rectangle* button on the *Life Science Applications* toolbar to do this.

5. When you have finished defining the ROIs, click the *New ROI - Rectangle* button on the *Life Science Applications* toolbar again.

Starting a FRET analysis

6. Activate one of the FRET images in the image window.

7. Click the *FRET Analysis* button located on the *Life Science Applications* toolbar.
   - The *FRET Analysis* dialog box opens.
8. The *FRET Analysis* dialog box offers different methods of computing the *Ffret*, *Fdon* and *Facc* source images in relation to each other.

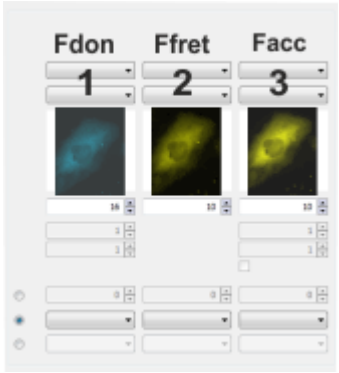
   Click this button to open an info window.
   - The info window displays all of the computation methods that are available in the *FRET Analysis* dialog box.
9. Select the *Gordon (1998)* entry in the *Method* list.
   - Gordon (1998):

     $$FRETN = \frac{MicroFRET}{Fdon * Facc * G}, \quad G = \frac{QY_a}{QY_d}\frac{\phi_a}{\phi_d}\frac{T_f}{T_d}$$

     The computation method was taken from the following publication:

     Gordon et al. 1998. Quantitative Fluorescence Resonance Energy Transfer Measurements Using Fluorescence Microscopy. Biophys. J. (74), 2702-2713
   - All of the loaded images that could be computed with the current image are listed in the *Image* list.

10. Enter the Gordon correction factor for the acquisition conditions and the fluorochrome that you are using in the *Correction G* field. If you don't know the Gordon correction factor, enter 1.
11. Select reference images **1**-**3**, described above, in the *Image* and *Channel* lists.

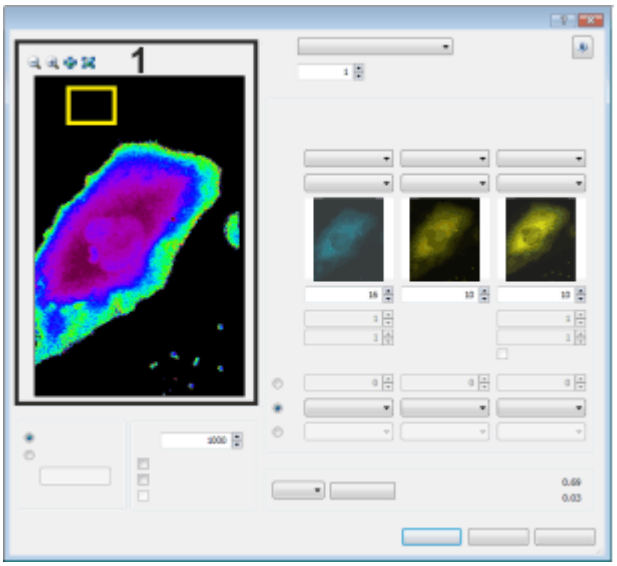


Select the appropriate FRET images.

12. Select the *Background > ROI* option. Select the ROI that you previously defined on the background of the image from each list.

Loading correction factors

13. Select the correction factors that you specified in the last step-by-step instructions for the reference sample from the *DBST/ASBT Correction Factors* list. In this example, the entry was *cfy-yfp*.
    - The correction factors that were loaded are now displayed in the *DSBT (Fdon)* and *ASBT (Facc)* fields.
    - The preview image in the *FRET Analysis* dialog box now shows the FRET image resulting from the Gordon method.
    - The FRET image resulting from the Gordon method is a gray-value image which automatically has a predefined pseudo color table applied to it in the preview window. High values are displayed in red with this pseudo color table and low ratio values are displayed in magenta.



The preview image (**1**) show the resulting FRET image as a pseudo color image. The ROI that was used for the background correction is shown in the preview image. Use the buttons above the preview image to change the preview image's zoom factor, if necessary.

14. If the resulting FRET image displays single pixels with high intensities in its background, this is image noise. In the *Thresholds* fields, increase the value until the image noise disappears.
15. Close the *FRET Analysis* dialog box with *OK*.
    - The resulting FRET image is added to the FRET image in an image layer. This means that the resulting image will be a multi-layer image.

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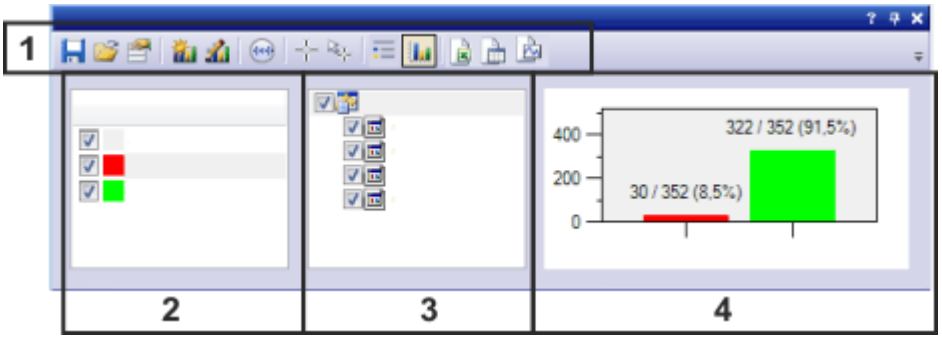
11. Measuring images

11.1. Tool Window - Object Counting



(**1**) Toolbar of the tool window
(**2**) Defining, selecting, and editing object classes
(**3**) Selecting images
(**4**) Viewing results

11.1.1. Toolbar of the tool window

	Saving, loading, and managing class definitions	You can save your objects' class definition to a parameter set. Then, the next time you want to count objects, you can simply load the object classes and use them again.
	Creating class	Click this button to create a new class.
	Editing class	Click this button to change the active class definition.
	Showing or hiding the digital reticle	Click this button to have a digital reticle displayed in the image window.
	Counting objects	Click this button to count objects.
	Editing objects	Click this button to correct a measurement.
	Switching between different results views	The results are displayed in the *Object Counting* tool window. Choose between a bar chart and a list view. Click the corresponding button to set the display you want.
	Exporting results	You can export the results to an MS-Excel file, a workbook or a chart.

Counting objects

1. Load the image you want to count objects on, or acquire an image. You can also count objects in live mode.
2. Select an object class in the *Object Counting* tool window or define one.
3. Click the *Count Objects* button to count objects.
   - When you count objects, you are in a special measurement mode. The button appears clicked, thereby showing you that the measurement mode is active. You can recognize this status by the button's colored background.
4. In measurement mode, you click on the objects in the active image that you want to count.
   - All counted objects are automatically assigned the active object class.

Note: In measurement mode, you can only count objects. In this mode, the majority of your software's other functions are not available.

To end measurement mode, release the *Count Objects* button. The measurement mode ends automatically when you define a new class or activate another image.

Editing objects

On the image, objects that have been counted are displayed with markers. You can delete or shift existing markers, for example, when you clicked the wrong object during counting.

Exporting results

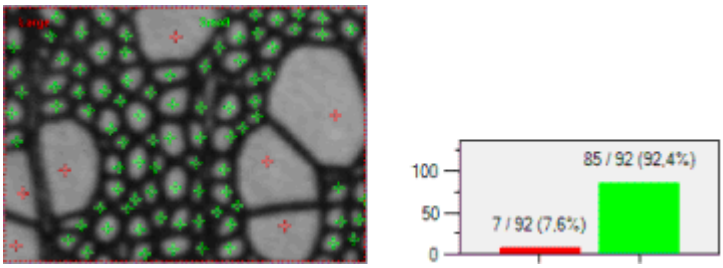
You can export the results to an MS-Excel file, a workbook or a chart.

11.1.2. Defining, selecting, and editing object classes

Use the *Classes* area in the *Object Counting* tool window for defining, selecting, and editing object classes.

What is an object class?
While counting, you can assign the objects the class you want. For example, if you want to count small and big objects on an image, define two classes.

Note: All classes you define are only valid for the image that is currently active in the image window. If you want to use classes for several images, save the class definition and load it again for the next image.



In the image shown, small and big objects have been counted. The green class was defined for the small objects. The red class was defined for the big objects. The bar chart shows the results. 7 big and 85 small objects have been counted on the image. 92% of all counted objects are small.

Defining object classes

A class is defined by a name and a color. All objects that belong to the same class are displayed in their class color, in the image and in the bar chart. The class name is used for the labels in the bar chart and for the results sheet. The class name is also shown in the image.

There are different ways in which you can define a class.

- In the *Object Counting* tool window's toolbar, click the *Create Class* button.
- Click the *<Enter class name>* entry, in the *Classes* area.
- Right click the *Classes* area of the tool window. From the context menu, select the *Create Class* command.
- Adopt a class definition from another image. To do so, select the image that contains the class definition you want, in the *Counting Documents* group, in the *Object Counting* tool window. Then select the *Copy Class Definitions to Active Document* context menu.

11.1.3. Selecting images

Use the part in the middle of the *Object Counting* tool window in order to manage images on which objects have already been counted.

List of all the measured images

All images on which objects have already been counted are listed below the *Counting Documents* entry, in the *Object Counting* tool window. The active image is shown in bold in the list.

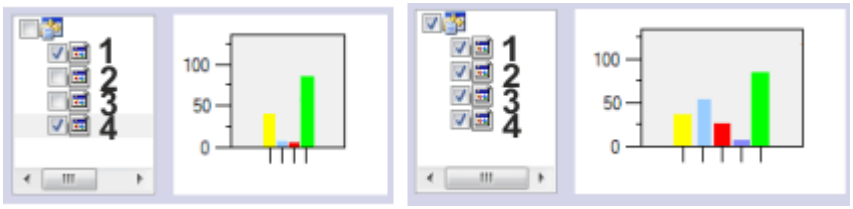
Showing and hiding results

Select the check box next to the image name in order to have the results shown in the results view, in the right part of the tool window.
Clear the check box next to an image name in order to have the corresponding results hidden in the results view.
Select the check box next to the topmost *Counting Documents* entry, to have the results for all images displayed at a time. Clear the check box to hide all results at a time.

When counting objects, the results of all selected images are added up.
When using the same object classes on several images, all objects that belong to this

object class are added up. When using different object classes, the results view shows all object classes that have been defined.

Note: In the *Classes* area, located on the left of the tool window, you only see the classes that have been defined for the active image. In the results view, in the right part of the tool window, all classes that have been defined on the selected images are shown.



In the example shown, objects have been counted on 4 images. On the left, only the results of the images (**1**) and (**4**) are taken into account. On the right, the results of all images are taken into account. You can see, that an additional object class has been counted on images (**2**) and (**3**).

11.1.4. Viewing results

The results are displayed in the *Object Counting* tool window. Choose between a bar chart and a list.

Which results are shown?

The results show the number of counted objects per object class. Additionally, the list view shows how many objects have been counted in total.

All images that are selected in the tool window's tree view are considered for the calculation of the measurement result.

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11.2. Interactive measurements

11.2.1. Overview

Your software offers a wide range of measurement functions. They enable you to quickly count objects and measure segments and areas. All the results will be saved together with the image and can also be output as a sheet.

Prerequisite

For making measurements, correctly calibrated images are an essential prerequisite.

Images that you have acquired with your software will have been automatically correctly calibrated when you have specified the objective you used. If your system has a motorized nosepiece or an encoder for the nosepiece, the correct magnification is automatically read-out before the image acquisition.

Should the image not yet have been calibrated, use the *Image > Calibrate Image* command to carry out a calibration.

Additional measurement functions in your software

In addition to the interactive measurement functions, your software offers you a further range of measurement functions.

Life Science Applications	The *Life Science Application* toolbar offers you various evaluation methods for your images.
Line Profile	Use the *Line Profile* tool window to measure the intensity profile along a line on an image.
Object Counting	Use the *Object Counting* tool window to manually count objects on your images.
	You can detect and analyze objects in images with your software.
Object Tracking	You can track and analyze the movement of objects with your software (cell movement for example).

Selecting the measurement environment

Measuring with help of the tool window

Switch to the *Count and Measure* layout when you want to measure images. You can find the *Measurement and ROI* tool window in the bottom section of this layout. In this tool window you have fast access to all measurement functions and settings which effect measurements. This tool window is at the same time the measurement display and contains all of the values that have been measured on the active image.

Note: Should, right at the bottom of the user interface, several tool windows lie one over the other, activate the *Measurement and ROI* tool window, by clicking on the

header of the *Measurement and ROI* tab. The tabs can be found under the tool windows.

Starting a measurement

Begin a measurement by selecting the measurement function you want. You can find the measurement function in the *Measurement and ROI* tool window, on the *Measurement and ROI* toolbar, or in the *Measure* menu.

Working in the measurement mode

As soon as you have clicked a measurement function, your software will automatically switch to a measurement mode. In the measurement mode, your mouse pointer will take on the shape of a cross on the image. A small icon indicating the selected measurement function attaches itself to the bottom right of the mouse pointer.

You can make as many measurements on the active image as you like using the measurement function that has been selected. The continuous measurement mode is valid for all loaded images. You can, therefore, easily measure numerous images one after the other.

The selected measurement function's button will keep its clicked appearance and in this way show you the current measurement function. You can recognize this status by the button's background color.

Finishing the measurement mode

You can explicitly switch off the measurement mode. To do this, click on the active measurement function's button again.

You automatically turn off the measurement mode when you switch to a different mouse pointer mode. For example, click the *Select Measurement Objects* button to switch to the selection mode. You can find the button either in the *Measurement and ROI* tool window or on the toolbar. You can select and edit measurement objects in this mouse pointer mode.

Changing the default measurement mode

The continuous measurement mode described above is preset by default. You can change this default setting. To do this, use the *Tools > Options* command. Select the *Measurement and ROI > General* entry in the tree view. Select the *Switch to 'Select Measurement Objects' mode after creating a measurement object* check box. Then, when you have completed a measurement, you will automatically leave the measurement mode again. This means you have to select the measurement function again before you start each interactive measurement.

Displaying and saving measurement results

The measurement results will be displayed directly on the image and in the *Measurement and ROI* tool window. Should this tool window not be visible, use the *View > Tool Windows > Measurement and ROI* command to display the tool window.

Saving the measurement results

The measurements will be saved along with the image, if you save the image in the TIF or VSI file format. You can, however, also export the measurement results in a results sheet, and save this as a file.

Showing and hiding measurement results in an image

The measurement results will be shown on the image in a special data layer, the measurement layer. On your monitor, image and measurement layer are shown together. The data of each, however, is individually stored if you use the TIF or VSI image file format. Try and picture the measurement layer as a transparency which is placed over the image. When you measure an image, the image data will not be changed by having the measurement results displayed on it.

You can, at any time, hide or show the measurement layers.
To do so, use the *Layers* tool window. There you have access to all of an image's layers. The eye icon identifies all of the layers that are currently on display on your monitor.
Click the eye icon in front of the *Measurement and ROI* layer to hide the measurements. Click an empty cell without an eye icon to make the corresponding layer reappear.

Editing measurements

You can edit existing measurement objects at any time. The measurement values in the *Measurement and ROI* tool window will be correspondingly updated.

Note: When you load an image file with measurement objects, it is only possible to edit the measurement objects if the image file has been saved in the TIF or VSI image file format.

Selecting measurement objects

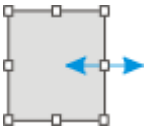
Before you can edit measurement objects, you have to select them. To do so, click the *Select Measurement Objects* button, and then select the measurement object(s). You can find the button either in the *Measurement and ROI* tool window or on the toolbar.

If the image is very large and many measurement objects have been defined, it can be difficult to find a particular measurement object in the image. In this case, select the measurement object that you are searching for in the *Measurement and ROI* tool window. Click your right mouse button and select the *Navigate to Measurement Object* command in the context menu. The measurement object you are looking for is then displayed in the image window.

Changing the position and size of measurement objects

You can move a whole measurement object while keeping the left mouse button pressed.

You can also change the size of a measurement object. Move the pointer onto a marker. By dragging the marker with the mouse button depressed, you can adjust the frame's size as wished.



Change the measurement object by moving the handles.

Deleting measurement objects

Click the [Del] key on your keyboard in order to delete the selected measurement object. You can select measurement objects that you want to delete in the image and also in the sheet in the *Measurement and ROI* tool window.

Changing the color, font, and line thickness of individual measurement objects

You can, at any time, change the color, font, and line thickness, of individual measurement objects. Select one or more measurement objects in an image and click your right mouse button to open a context menu. In the context menu you'll find the following commands. You can use them to change the appearance of the selected measurement objects.

Recolor automatically
Change Color
Helper Lines
Change Line Thickness
Adjust Position
Change Font

Measuring in the live mode

All of the measurement functions are also available in the live-image. You can therefore, e.g., quickly measure a segment in the live-image.

When you finish the live mode with the *Acquire > Snapshot* command, the measurements that you carried out in the live-image are applied to the image that was acquired.

Measuring on different image and document types

Measuring on image series

You can combine a series of individual images into one image. What results is a time stack in which all of the frames have been acquired at different times, for example.

You can make measurements on every frame. Display the required frame on your monitor. To do this, use the navigation bar in the image window. Then carry out the measurement on this frame. The measurement will be permanently linked to this frame, i.e., the measurement will only be displayed on your monitor when the frame on which you made this measurement is also on display.
The measurement results will be shown in the *Measurement and ROI* tool window. You can give every measurement the number of the frame on which it was made. For time stacks, for example, you can do this using the *Index (t)* measurement parameter.

Measuring on multi-channel images

A multi-channel image is made up of individual fluorescence images. For multi-channel images you can choose to measure on each fluorescence image separately or to define one measurement object for all color channels simultaneously.

Clear the *Tools > Options > Measurement and ROI > General > Measure on all channels* check box.
Now, you will measure on each fluorescence image separately. To do so, set up the color channel you want on your monitor. To do this, use the navigation bar in the image window. Then carry out the measurement on this image. The measurement will be permanently linked to this color channel, i.e., the measurement will only be displayed on your monitor when the color channel on which you made this measurement is also on display.
The measurement results will be shown in the *Measurement and ROI* tool window. You can give every measurement the name of the color channel on which it was made. To do this, use the *Channel* measurement parameter.

Select the *Tools > Options > Measurement and ROI > General > Measure on all channels* check box.
Now, each measurement object you define will be measured on each color channel. All measurement results will be shown in the *Measurement and ROI* tool window.

Measuring on multi-layer images

With some functions, e.g., with the *Image > Combine Color Images* function, a multi-layer image will be created. This multi-layer image is made up of several layers.

Measurements always apply to one image layer. For this purpose, show the image layer on your monitor, on which you want to make measurements. To do so, use the *Layers* tool window. Then carry out the measurement on this image layer. The measurement will be permanently linked to this image layer, i.e., the measurement will only be displayed on your monitor when the image layer on which you made this measurement is also on display.
The measurement results will be shown in the *Measurement and ROI* tool window. You can give every measurement the name of the image layer on which it was made. To do this, use the *Layer* measurement parameter.

Measuring on kymograms

Use the *Kymograph* tool window to create a visual representation of the movement of objects. The source image is usually a time stack. The result is a kymogram. The kymogram is an image that is calibrated differently along it's horizontal and vertical axes. For example, the X-direction is calibrated in units of length and the Y-direction is calibrated in units of time.

Use the *Kymogram Polyline* measurement function to make measurements on a kymogram. This measurement function doesn't deliver any results for other image types.

Measuring on charts

Your software has its own chart document. A chart can be saved, edited and also measured.

Use the *Line Profile* tool window to measure the intensity profile along a line on an image. In the tool window, click the *Export to Chart* button to export the line profile to a chart.

As soon as a chart has become active in the document group, the *Measurement and ROI* tool window changes its appearance. From then on, only the measurement functions that you can use for charts are available.

	Name of the button	Description
	Horizontal Line	In a chart, measure the horizontal distance between two interactively determined points.
	Multiple Horizontal Lines	In a chart, measure the horizontal distance between a reference line and an interactively determined point.

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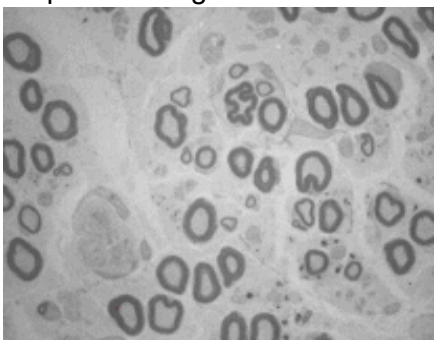
11.2.2. Measuring image objects interactively

Task: You want to measure the diameter of some cells.
To do this, load a suitable image, or acquire one. Measure the diameter of some cells. Then edit the measurement and delete some of the measurements that have been performed. Enter the results in a MS-Excel sheet.

1. Use the *View > Tool Windows > Measurement and ROI* command to display the *Measurement and ROI* tool window.
   - You'll find the tool window at the lower edge of the user interface. It could be under another tool window. If this is the case, click the *Measurement and ROI* tab at the bottom of the user interface to bring the tool window into the foreground.

Loading an image

2. Acquire an image or load one.



   - During the installation of your software some sample images have been installed, too. You can follow these step-by-step instructions for measuring images when you use the exemplary image Neurons.tif.

Setting the labeling color

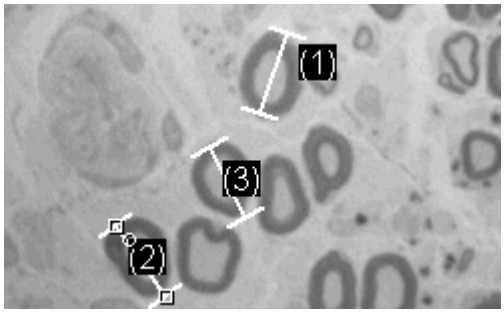
The measurement results will, in accord with the default settings, be written in red in the image, without a background. This can be hard to read on some images. Change the labeling settings.

3. Use the *Tools > Options* command.
4. Click the *Measurement and ROI > Measurement Display* entry in the tree view.
5. Click in the *Background Color* field and choose a color, for example, black.
6. Select the *Text color > Fixed colors* option and select a suitable color from the palette. Select the color white, for example, to display the measurements in white and the labels in white on a black background.
7. Close the dialog box with *OK*.

Measuring lengths

8. Click the *Arbitrary Line* button, located on the toolbar at the top of the tool window.
9. Click with your left mouse button at the starting point and end point of the reference distance.
10. If you have measured a reference distance, you can immediately proceed with the next measurement.

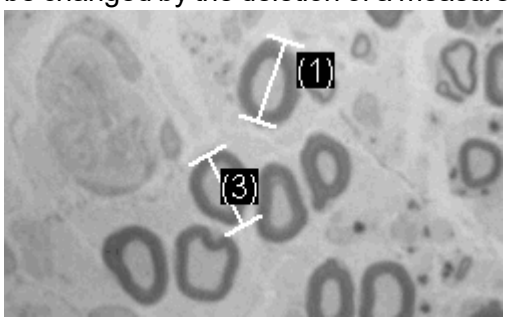
11. Click the *Arbitrary Line* button again to end the length measurement.
12. Take a look at the results in the tool window and in the image.
    - The illustration shows the image with three executed measurements. Measurement 2 has been selected.



Deleting measurements

13. Click one of the measurement results in the *Measurement and ROI* tool window.
    - The corresponding line will be selected in the image.
14. Press the [Del] key.
    - The measurement will be deleted both in the image and in the tool window.
    - When a measurement has been deleted, the image and the tool window contain one measurement less. The IDs of the remaining measurements won't

be changed by the deletion of a measurement.



Note: When you've finished making the measurement, you should switch off the measurement mode, since you could otherwise accidentally select your measurements and move them.

15. Check whether one of the buttons on the *Measurement and ROI* tool window's toolbar appears clicked. Release this button

Exporting results to MS-Excel

16. Click the *Export to Excel* button.
17. In the In/Output dialog box you set up the directory in which the data is to be saved, and enter the name of the MS-Excel sheet. Adopt the *Excel-Sheet (*.xlsx)* file type.
18. Click the *Save* button to have the MS-Excel sheet with the measurement results saved.

Closing the image

19. Click the button with the cross [x] to the right of the image name in the document group.
    - You have changed the image because you've added interactive measurements. For this reason, you'll receive a query whether you wish to save the image or not.
20. Save the image in the TIF or VSI file format. The measurements will then also be saved in the image file. They can at any time, be edited deleted or augmented.

11.2.3. Outputting various measurement parameters

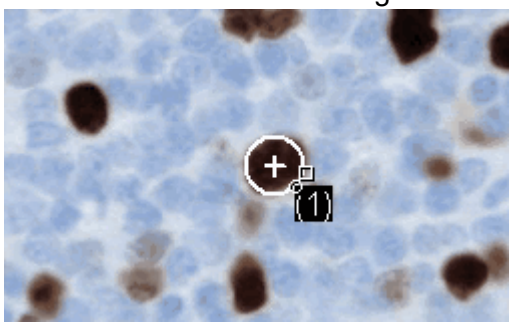
Task: You want to measure some cells. Measure the cell as a circular object. Have a variety of measurement parameters, such as the area, the perimeter and the diameter, output. Have the diameter shown in the image.

1. Acquire or load an image, for example, the BadTissue.tif example image.

Measuring areas

2. In the *Measurement and ROI* tool window, click the *2 Point Circle* button.
3. Left click the center point of a cell that you want to measure.
4. Move your mouse, and in the process drag out the circle. Match the circular object as well as possible to the cell. Click the left mouse button.

5. Click the *2 Point Circle* button again, and switch off the measurement mode.
6. Take a look at the result in the *Measurement and ROI* tool window.
   - The illustration shows the image with a circle measured.

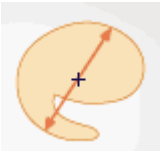


Viewing the list of measurement parameters

7. In the *Measurement and ROI* tool window, click the *Select Measurements* button.
   - In the dialog box you'll see a list with all of the available measurement parameters. At the bottom of the dialog box you'll see a list of the measurement parameters that are currently calculated for all objects.

Outputting additional measurement parameters

8. Go to the list of all of the available parameters, then click the *Diameter* measurement parameter.
   - On the right, an illustration shows you how the parameter is calculated.



   You can see that there are different ways in which the diameter of a 2D object can be calculated.
9. Click the *Mean* entry in the list under the illustration, to select the *Mean (Diameter)* measurement parameter. When you do this, the mean value of all of the possible diameters is determined.
10. Click the *Add 'Mean (Diameter)'* button.
    - This measurement parameter will be added to the list of measurement parameters to be calculated. All of these measurement parameters will be displayed in the tool window.
11. Close the dialog box with *OK*.
12. Take a look at the result for the circle's diameter in the *Measurement and ROI* tool window.

Outputting measurement parameters in the image

13. Open the *Select Measurements* dialog box.
14. At the bottom of the list of all of the calculated measurement parameters, click the *Mean (Diameter)* measurement parameter.

15. To the right of this list you'll see a button with a blue arrow . Click this button to move the measurement parameter to the top of the list.

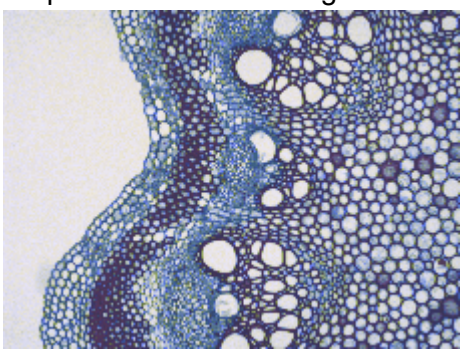
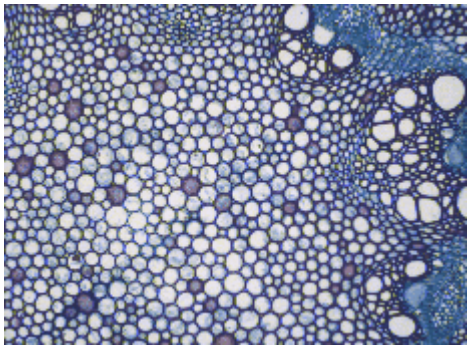
16. Close the dialog box with *OK*.
17. Take a look at the result for the circle's diameter in the image.

11.2.4. Measuring several images

Task: You want to measure cells on multiple images. To do so, acquire some images and measure them one after another. Have the results from all images displayed simultaneously. Take a look at the mean value for all of the measurements.

Loading images

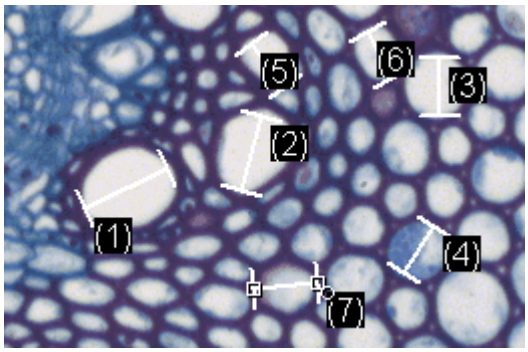
1. Acquire or load some images.

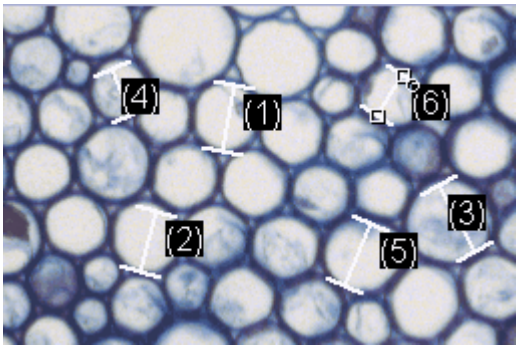



- During the installation of your software some sample images have been installed, too. You can carry out these step-by-step instructions using the Clematis04.tif and Clematis05.tif example images.

Measuring cells

2. Activate the first image in the document group.
3. Click the *Arbitrary Line* button, located on the toolbar at the top of the *Measurement and ROI* tool window. Measure the diameter of several cells.
4. Activate the next image. Measure the diameter of several cells on this image, too.
5. Click the *Arbitrary Line* button again, and switch off the length measurement.
   - Cells have been measured on both images.





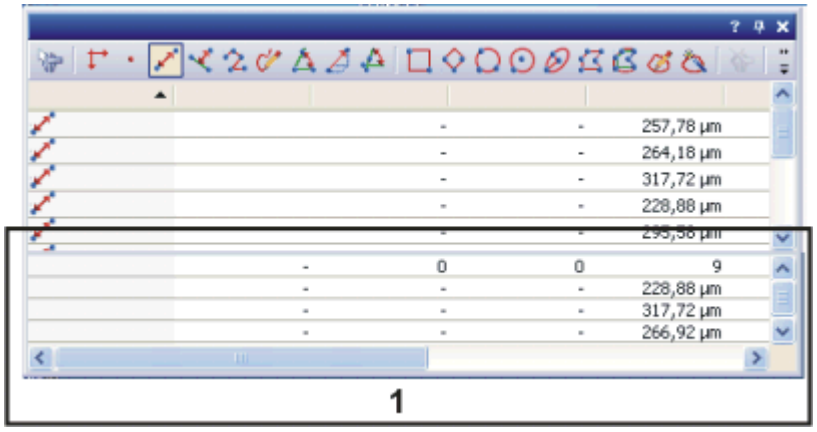
Displaying the measurement results of all of the images

6. In the *Measurement and ROI* tool window, click the *Measurement and ROI Options* button.
7. Select the *Measurement and ROI > Results* entry in the tree view.

8. Clear the *Show measurement objects > Only of the active image* check box.
9. Close the dialog box with *OK*.
   - Now, the results for both images will be shown simultaneously in the tool window.
   - Use the *Document* measurement parameter to display the name of the image with which the measurement results are associated in the results sheet. Now, you can match the measurement results unambiguously to an image, even if all measurement results are displayed together in the tool window.

Viewing statistical parameters

10. In the *Measurement and ROI* tool window, click the *Measurement and ROI Options* button.
11. Select the *Measurement and ROI > Results* entry in the tree view.
    - In the *Statistics* group, you can find various statistical parameters.
12. Select the *Mean* check box.
13. Close the dialog box with *OK*.
    - Now, in the *Measurement and ROI* tool window under the measurement results, the chosen statistical parameter (**1**) will by shown. You can see there the mean value of the layer thickness for all of the measured images.



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11.3. Line profile

Use the *Line Profile* tool window to create one or more line profiles on an image. A line profile measures the intensity along a particular line in an image.

11.3.1. Creating line profiles

Task: Let's assume you want to measure the intensity profile of each cell in a multi-channel fluorescence image. To do this, create a line profile that includes as many cells as possible.

1. Load the HER2 (3x16-bit).tif example image.

Checking the default settings

2. Use the *Tools > Options* command and select the *Line Profile > New Line Profiles* entry in the tree view.
3. Click the *Default* button to select the default settings.

   Fixed colors for the lines are set by default. Select a suitable color from the palette next to the *Fixed colors* option. You can select white for example.
4. Close the *Options* dialog box with *OK*.

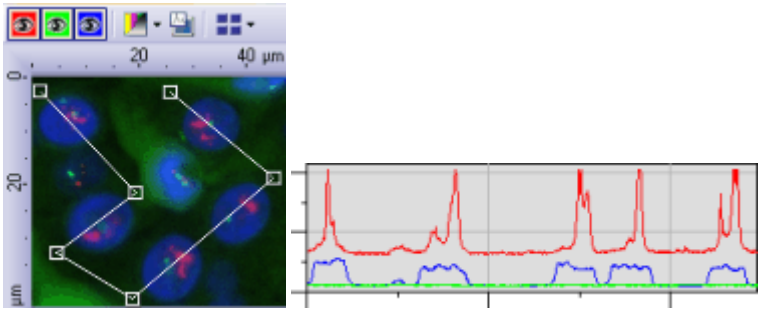
Creating a line profile

5. Use the *View > Tool Windows > Line Profile* command to display the *Line Profile* tool window.
   - No line profile has been defined on the image yet. This is why the tool window is empty.

6. Click the *Profile of Polyline* button on the tool window's toolbar.
   - The mouse pointer will change its shape when you move it on the image window.
7. Define a line on the image by left clicking. Click the right mouse button to finish defining the line.

8. Click the small arrow next to one of these buttons .

   Select the *Show Profiles in Colors of Channels* command to use the channel colors for the line profiles. You can find this button on the *Line Profile* tool window's toolbar.
   - In the *Line Profile* tool window, a line profile is displayed for each color channel. The colors of the lines correspond to the colors of the color channels.



In the image on the left, you can see the line along which the line profile is measured. On the right you can see three line profiles for the line that has been defined. A separate line profile is displayed for each color channel.

Displaying line profiles for individual color channels

- 

  A navigation bar is displayed in the image window. It contains a button for each channel to enable you to display or hide that channel. The eye icon indicates that the channel is currently visible.

9. Click the color channel button in the navigation bar to have a color channel displayed or hidden. You can hide the green and the blue color channels, for example.
   - Only the line profile for the red color channel is now still shown in the *Line Profile* tool window.

Exporting results to MS-Excel

10. In the *Line Profile* tool window, click the *Export to Excel* button.
11. In the In/Output dialog box you set up the directory in which the data is to be saved, and enter the name of the MS-Excel sheet. Adopt the *Excel-Sheet (*.xlsx)* file type.
12. Click the *Save* button to have the MS-Excel sheet with the measurement results saved.
    - In the sheet you will see the line profile for all of the color channels that are currently visible in the image window.

Closing the image

13. Click the button with the cross [x] to the right of the image name in the document group.
    - You have changed the image by adding a line profile. For this reason, you'll receive a query whether you wish to save the image or not.
14. Save the image in the TIF or VSI file format. The line profile will then also be saved in the image file. The line profile can at any time be edited, deleted or expanded.

11.3.2. Creating more than one line profile

Task: Let's assume you want to measure the intensity profile of each cell in a multi-channel fluorescence image. To do this, create a line profile for each cell.

1. Load the HER2 (3x16-bit).tif example image.

Checking the default settings

2. Use the *Tools > Options* command and select the *Line Profile > New Line Profiles* entry in the tree view.
3. Increase the value in the *Averaging* field. Enter a value of 10, for example.
   - Now the line profile will be averaged over a specific image area.
4. Close the *Options* dialog box with *OK*.

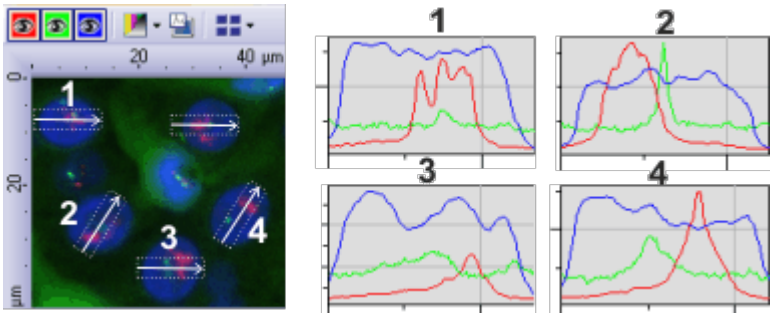
Creating a line profile

5. Use the *View > Tool Windows > Line Profile* command to display the *Line Profile* tool window.
6.  Click the *Profile of Arbitrary Line* button on the tool window's toolbar.
   - The mouse pointer will change its shape when you move it on the image window.
7. Define a line across each cell in the image. You define the line by clicking its start and end points.
   - The line now has the width that you set in the options. The width of the line is shown using a dashed rectangle.
   - Now when the line profile is computed, all of the pixels that are perpendicular to the actual line will be averaged.
   - In the *Line Profile* tool window, a line profile is displayed for each color channel. The colors of the lines correspond to the colors of the color channels.

Displaying more than one line profile in the tool window

- By default, only one chart is displayed in the *Line Profile* tool window.

8.  In the *Line Profile* tool window, click the *Arrange Charts* button.
   - The *Arrange Charts* dialog box opens.
9. Define a layout for the chart area in the *Line Profile* tool window. To do this, select the required cells in the grid. You could specify that 4 charts should be displayed in an arrangement of 2x2, for example.
   - You now see several charts in the *Line Profile* tool window. Three line profiles are displayed in each chart, one for each color channel.



In the image on the left, you can see four lines along which the line profile is measured. On the right you can see the charts with the corresponding line profiles.

Exporting the results to a workbook

10. In the *Line Profile* tool window, click the *Export to Workbook* button.
    - A new workbook is created in your software's document group.
    - A separate worksheet is created for each line profile that has been measured on the active image. Each worksheet contains the data for each of the line profiles for the color channels. No line profiles will be created or exported for color channels that are currently not shown in the image window.

11.3.3. Showing and hiding line profiles

You can hide or show the line profiles in the image at any time.

1. Load an image on which line profiles have been defined.
   - When you load the image the line profiles are not visible at first.
2. In the *Line Profile* tool window, click the *Show Line Profiles* button.
   - Now the lines along which the line profile was measured are shown in the image. You can see the line profiles in the *Line Profile* tool window.
   - If no line profile has yet been measured on the active image, a line now appears in the image window. At the same time, you see the current line profile in the *Line Profile* tool window.

11.3.4. Editing existing line profiles

You can move the line along which the line profile is measured. You can also change its length, course, and color. You can smooth the line profile.

1. Load an image on which line profiles have been defined.

Editing lines

2. Click the *Select Profile Line Objects* button, and select the line that you want to edit in the image. You can find this button on the *Line Profile* tool window's toolbar.
3. Drag the whole line to a different position.
   - Each line contains small square handles.
4. You can change the course of a line by moving the handles.

Editing line profiles

5. Select a line in the image. Click the right mouse button to open a context menu.

6. Use the *Line Profile Properties* command to change the settings for the line profile.

7. Click the small arrow next to one of these buttons .

   Select the *Show Profiles in Colors of Lines* command to display the line profiles in the color of the line that has been defined in the image. You can find this button on the *Line Profile* tool window's toolbar.

8. Use the *Change color* command in the context menu to select a new color for the selected line. The color of the line profile in the tool window will also be changed if the line profiles are being displayed in the colors of the lines.

Copying line profiles to a different image

9. Select a line in image 1.
10. Select the *Copy* command from the context menu.
11. Go to a different image, image 2, and use the [Ctrl + V] shortcut.
    - The line will be copied to the same position in image 2 as it occupies in image 1.
    - In image 2 the line profile will be measured and displayed in the *Line Profile* tool window.

Note: A line can only be copied to a different image if it will fit. If image 2 is smaller than image 1, the source image, the *Paste* command will not be available.

11.3.5. Deleting line profiles

1. Load an image on which line profiles have been defined.

2. Click the *Select Profile Line Objects* button, and select the lines that you want to delete in the image. You can find this button on the *Line Profile* tool window's toolbar.
3. Click the right mouse button to open a context menu.

4. Select the *Delete Line Profile* command from the context menu.
   - The selected lines will be deleted from the image.
   - The corresponding line profiles will be deleted from the *Line Profile* tool window.
   - This doesn't change the numbering of the remaining line profiles.
   - The arrangement of the charts in the *Line Profile* tool window changes automatically to make best use of the space in the tool window.

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12. Automatic image analysis

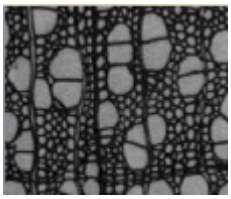
12.1. Automatic object analysis

You can use an automatic image analysis to carry out numerous measurement tasks. Several typical tasks and their process flow are described here.

Note: Your software offers two different software packages for automatic object analysis. In the basic version, not all of the described functions are available. You can find more information in the individual step-by-step instructions.

12.1.1. Counting objects

Task: You have an image with objects that interest you. You want to know how many of these objects there are in the image.



You want to detect and count wood cells in the example image.

Preconditions

The objects that you want to count must not be connected, but must be clearly separated from one another. The objects in the foreground should be optically clearly separated from the image's background. In the example image shown, the background is dark. The objects lie in the foreground and are light in color.

Preparations

1. Use the *View > Tool Windows > Count and Measure* command to have the *Count and Measure* tool window displayed.
2. Acquire an image or load one.
   - During the installation of your software some sample images have been installed, too. You can follow these step-by-step instructions using the *WoodVessels.tif* example image.

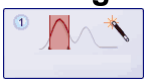
Setting options

3. Open the *Options* dialog box by clicking the *Count and Measure Options* button, located in the *Count and Measure* tool window.
4. Select the *Count and Measure > Detection* entry in the tree view.
5. In the *Options* group, enter the value 5 in the *Minimum object size* field. An object must now be at least 5 pixels large in order to be counted as an object. By doing that, you will rule out the possibility that individual pixels, that may well have the same color or intensity as the objects, but don't belong to an object, are counted as objects, which would then falsify the results. This way you can exclude noise

and dust particles.

6. Click *OK* to exit the *Options* dialog box.

Setting threshold values



7. In the *Count and Measure* tool window, click the *Automatic Threshold* button to open the *Automatic Threshold* dialog box.
   - If the *Automatic Threshold* button is not yet active, activate it first. To do so, select the *Automatic Threshold* entry in the *Threshold* button's menu. You open this menu by clicking the small arrow next to the button.
   - The threshold values are set automatically in the *Automatic Threshold* dialog box.
   - All of the objects that have been detected will be displayed in color.
8. Check whether the objects have been correctly detected.

   Should the objects not have been correctly recognized, go to the *Background* group and enter whether the background is bright or dark.

   Select e.g., for the image shown above, the *Background > Dark* option, since the image shows bright objects against a dark background.

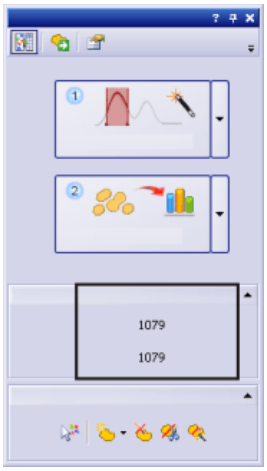
9. Only if the *Remove Phase* button in the *Phase thresholds for ...* group is active:

   Delete all but one of the phases by continuing to click the *Remove Phase* button until the button becomes inactive.
   - By doing that, you will make certain that no phases from earlier analyses are still defined.

Viewing the results

10. To obtain the results, click the *Count and Measure* button in the *Automatic Threshold* dialog box.
    - The *Automatic Threshold* dialog box is closed.
    - The number of objects found is displayed in the *Object Count* group in the *Count and Measure* tool window.
    - The objects that have been analyzed are then displayed in color, on their own image layer. This image layer is called *Detected Objects*. Use the *Layers* tool window to make these image layers appear or disappear, or to delete them.

Prerequisite: You are working with the *Count & Measure Full* solution. In the basic version, the following functions are not available:



The number of objects detected will be shown below, in the *Count and Measure* tool window, in the *Object Count* group. Should you not be able to see this number, click the small black arrow to make it visible.

Separating objects

Prerequisite: You are working with the *Count & Measure Full* solution. In the basic version you can't edit objects.

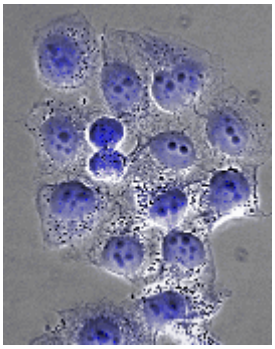
It is sometimes the case that two objects that are next to each other are not detected separately because, as far as the software is concerned, they are joined together. These sorts of objects can be separated manually.

1. Zoom into the image to enable you to better process the object.
2. Then click the *Manually Split Objects* button, located in the *Edit Objects* group, then move your mouse pointer onto the image.
3. Now define a separation line through the object by clicking the left mouse button. Make sure, when you do this, that you drag the line over the object's outside edge, since otherwise it won't be separated.
4. Right click to confirm the separation line.
   - The object will then be divided up into two independent objects. The results will be updated.
5. Then click the *Manually Split Objects* button again, located in the *Edit Objects* group, to leave the mode for splitting objects.

Left: Two objects are touching each other and thus are counted as a single object.
Middle: Draw a separation line through the object.
Right: The joined up object has been separated, there are now two independent objects.

12.1.2. Counting objects that belong to different phases (setting threshold values)

Task: You have an image on which you define two phases. You want to know how many objects there are per phase, in the image.



In the image, two phases are to be defined. The first phase is to map the black objects within the blue, round object. The second phase is to map the blue, round objects.

Preconditions

The objects that you want to count must not be connected, but must be clearly separated from one another. The objects in both of the phases must have different intensity values by which one can differentiate between them.

Setting options

1. In the *Count and Measure* tool window, click this button to open the *Options* dialog box.
2. Select the *Count and Measure > Detection* entry in the tree view.
3. In the *Options* group, enter the value 5 in the *Minimum object size* field to specify the minimum object size. By doing that, you rule out the possibility that individual pixels, that may well belong to the phase, but not to an object, are counted as objects, which would then falsify the results.
4. Select the *Count and Measure > Measurements* entry in the tree view.
   - In the basic version:
     From the *Class Measurements* list, select the *Object Count* and *Object Class* entries.
   - With the *Count & Measure Full* solution:

     Click the *Select Class Measurements* button located in the *Measurements* group. In the *Select Class Measurements* dialog box, add the *Object Count* and *Object Class* measurement parameters and close the dialog box.
5. Click *OK* to exit the *Options* dialog box.

Setting threshold values

6. In the *Count and Measure* tool window, click the *Manual Threshold* button to open the *Manual Threshold* dialog box.
   - If the *Manual Threshold* button is not yet active, activate it first. To do so, select the *Manual Threshold* entry, in the *Threshold* button's context menu. You open

this menu by clicking the small arrow next to the button.

7. Only when the *Remove Phase* button in the *Phase* group is active:
   Delete all but one of the phases by continuing to click the *Remove Phase* button until the button becomes inactive.
   - By doing that, you will make certain that no phases from earlier analyses are still defined.
8. Double click the *Phase Name* field and assign a name for the first phase. Click any position outside this field, or click the [Enter] key to leave the field again.
   - The first phase in the *Phase thresholds for channel '...'* group will be automatically selected.

9. Click the *New Threshold* button to set an initial value for the selected phase's threshold value range.
   - As soon as you move your mouse pointer onto the image it will change its shape to that of a pipette.

Note: Always start by defining the darkest phase, the one with the smallest threshold values.

10. Click on one pixel or on the image area whose intensity value is to be utilized as the initial value for the threshold range.

   - Once the initial value has been set, your mouse pointer will automatically change into a pipette with plus icon .
11. Then, continue clicking pixels that are typical of the first phase, until the required structures in the image are a part of the phase.

12. Should too many pixels have been selected, click the *Shrink Threshold* button to have these pixels excluded from the phase again.
   - The threshold value range will continue to be reduced until it no longer contains the pixels you have selected.

   - Alternatively, click the *Undo Pipet* button.

13. Click the *Add Phase* button to add the second phase, then proceed exactly as you did for the first phase.

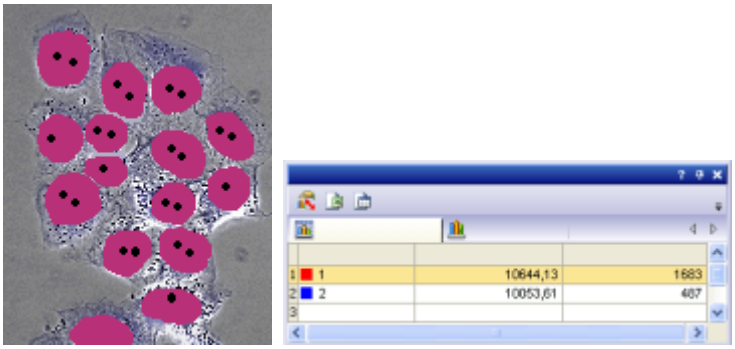
Selecting the classification

14. In the *Count and Measure* tool window, click this button to open the *Options* dialog box.
15. Select the *Count and Measure > Classification* entry in the tree view.
16. Select the *Phase* classification. Now, all of the objects belonging to one phase will also belong to one class.
17. Close the dialog box with *OK*.

Viewing the results

18. To obtain the results, click the *Count and Measure* button in the *Manual Threshold* dialog box.
   - The *Manual Threshold* dialog box is closed.

19. Open the *Count and Measure Results* tool window by using the *View > Tool Windows > Count and Measure Results* command.

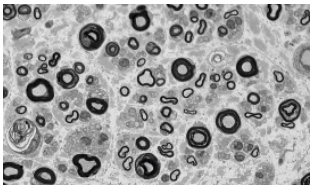


The total number of objects detected in all of the phases will be shown below, in the *Count and Measure* tool window, in the *Object Count* group. The results for the *Object Class* and *Object Count* measurement parameters will be displayed in the results sheet. The *Object Count* measurement parameter now displays the number of objects that belong to a phase. The *Object Class* measurement parameter enables you to include the name and the color of the phase in the results table. You can compare the results for both of the phases directly with each other.

12.1.3. Measuring objects (selecting and outputting measurement parameters)

Prerequisite: You are working with the *Count & Measure Full* solution. In the basic version you can't measure individual objects.

Task: You have an image with objects of different sizes. You want to know the area of the largest object and to have a close look at that object in the image. In addition to that, you want to export the results into a sheet.



Preparations

1. Acquire or load an image.
2. Carry out an automatic object analysis on the image.

Selecting a measurement parameter

3. Open the *Options* dialog box by clicking the *Count and Measure Options* button, located in the *Count and Measure* tool window.
4. In the tree view, select the *Count and Measure > Measurements* entry, then click the *Select Object Measurements* button, located in the *Measurements* group.
5. In the *Select Object Measurements* dialog box, add the *Area* and *Object ID* measurement parameters and close any open dialog boxes.
   - From some measurement parameters other, more complex, measurement parameters can be derived. In this case, you will find the basic measurement parameters in the list of measurement parameters. Select the basis

measurement parameter from the list and define which measurement parameters are to be derived from it in the area of the dialog box to the right of the list.
There are, for example, many different ways of determining the inner extent of objects. In this case, you can select between the minimal, maximal and mean inner extent.

6. Next, in the *Count and Measure* tool window, click the *Count and Measure* button to output the results.

Viewing and sorting the results

7. In the *Count and Measure Results* tool window, select the *Object Measurements* results view.
   - The measurement values for the objects' areas are displayed in the *Area* column.
8. Sort the *Area* column to find out which value is the smallest or the largest. To do so, double click on the header of the *Area* column.
   - This column's measurement values will then be sorted in ascending or descending order.
9. Double click the header of the column again to sort the measurement values in the reverse order.
   - An arrow in the header will show you the direction in which they are sorted.

Object - sheet link

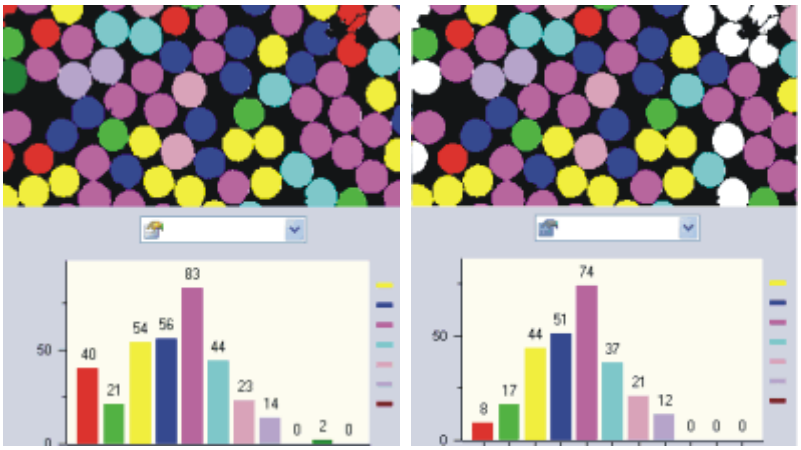
10. Select the largest value in the *Area* column.
    - The corresponding object will likewise be selected in the image window. In this way, you can easily find an object that belongs to a specific value, and view it.
11.  In the *Object Measurements* results view, click the *Export to Excel* button.
12. In the *Export Measurements* dialog box, assign the sheet a significant name, then save the sheet in the required directory.

12.1.4. Filtering objects

Prerequisite: You are working with the *Count & Measure Full* solution. In the basic version you can't filter objects.

Objects that disturb you, or that don't interest you, can be excluded from the measurement results. All of the measurement values that lie outside the defined measurement value area, won't be displayed, nor taken into account in any of the results views.

Task: On an image with spheres of different sizes, 9 size classes are defined. You want to know how many spheres fall into which size class. When the analysis has been carried out, you discover that the number of the small spheres has been overestimated, because spheres that weren't correctly separated, were also taken into account (image on the left). Define an object filter that only counts roughly circular objects.



Left: At the top right of the image, you can see some spheres that weren't divided properly. They have been sorted into the class of small spheres and are displayed in red.
Right: After the definition of an object filter, the number of objects in each class has changed. In particular, the red class of small spheres now has fewer objects.

Preparations

1. Load the image you want to analyze or acquire one.
2. Carry out an automatic object analysis on the image.
3. In the *Count and Measure Results* tool window, switch to the *Object Filter* results view.
   - In the table you will see a list of all of the selected measurement parameters and their corresponding filter ranges. There will always only be one measurement parameter active.

- If the measurement parameter that you want to use for the object filter doesn't appear in the list, click the *Select Object Measurements* button. You can find the button on the *Count and Measure Results* tool window's toolbar.
  If you only want to evaluate the almost spherical objects, you can select the *Sphericity* object parameter.

Entering the filter range directly

4. In the table in the *Object Filter* results view, click the measurement parameter for which you want to define a filter range.
5. Double click in the *[Min.* field, located next to the measurement parameter to enter the lower value for the filter range.
6. Either enter the required measurement value directly, or use the arrow keys.
7. Double click in the *Max.[* field, then enter the higher value for the filter range.
   - The higher value itself no longer belongs to the filter range.
   - You can delete individual values by double clicking the value, then pressing the [Del] key.

Defining the filter range interactively

8. In the table, click the measurement parameter for which you want to define a filter range.

9. Click the *Select minimum value* button, above the *Measurement* list to define the filter range's lower value.
   - The mouse pointer will change its form.
10. Click an object whose measurement value is to be used as the lower value for the filter range.
    - The measurement value will then be automatically adopted in the *[Min.* field. When you, for example, define a filter range for the *Area* parameter, click the smallest object that you still want to measure.
    - In the image window, the result of the filtering of the objects can be seen straight away. All of the values that are outside the defined filter range will be excluded from the results.
    - The filter range contains precisely those values that are to appear in the measurement results. All of the values that are outside the defined filter range will be excluded from the results.

    - The *Toggle Object Filter* button appears clicked, thereby showing you that the object filter is active.

11. If you want to undo the selection you've made, click the *Clear minimum value* button.

12. Click the *Select maximum value* button to define the filter range's upper value.
13. Click an object whose measurement value is to be used as the upper value for the filter range. Click the largest object that you still want to measure.
    - The measurement value is rounded up and automatically adopted in the *Max.[* field. The object that has been clicked is still within the filter range.

Switching off the object filter

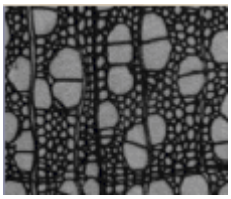
14. Release the *Toggle Object Filter* button.
    - Now all of the objects that have been detected will be taken into account again.
    - Click the *Toggle Object Filter* button again to switch the last object filter on again.

Note: A defined object filter is not automatically deactivated when you load another image. If, for example, no objects are shown, make sure that the object filter is deactivated.

12.1.5. Classifying objects

Prerequisite: You are working with the *Count & Measure Full* solution. In the basic version, you can't define your own classifications.

Task: You have an image with two object classes, e.g., large and small cells. You want to know how many objects fall into which size class.



Preparations

1. Acquire an image or load one. You can follow these step-by-step instructions using the *WoodVessels.tif* example image.
2. Perform an automatic object analysis on the image.
3. Select the *Area* object measurement.

Selecting measurement parameters for the object classes

4. In the *Count and Measure Results* tool window, select the *Class Measurements* results view.
5. Click the *Select Class Measurements* button, then in the *Select Class Measurements* dialog box, add the *Mean (Area)*, *Object Class* and *Object Count* measurement parameters.
   - With the *Mean (Area)* parameter, the mean area of all of the objects in a class will be calculated. That's to say, the parameter give you a measured value for how large the objects in this class are, on average.
   - With the *Object Class* parameter, you write the name and the color of the class in the results sheet, as well. You should, without fail, adopt this parameter in the results sheet to make it possible to assign the measurement results correctly to the individual classes. You can also adopt this parameter in the *Object Measurements* results sheet. Then, in the results sheet, you'll be able to immediately recognize to which class each of the individual objects belongs.
   - At the end, the *Object Count* parameter delivers the values you are looking for in the task: the number of objects found in each class.
6. Close the *Select Class Measurements* dialog box.

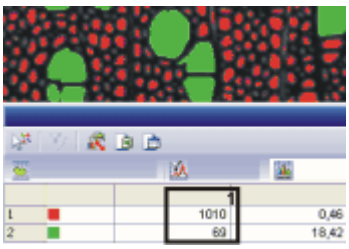
Defining classes

7. Open the *Options* dialog box by clicking the *Count and Measure Options* button, located in the *Count and Measure* tool window.
8. Select the *Count and Measure > Classification* entry in the tree view.
9. In the *Current Classification* group, click the *New Classification* button, then select the *New 'One parameter Classification'* entry.
   - The *Define 'One parameter' Classification* dialog box opens.

10. Enter a descriptive name for the new classification in the *Name* field, *size class* for example.
11. Select the *Area* entry in the *Measurement* list.
    - Only the measurement parameters that have been selected for the object analysis are shown in the list.
12. Click the *Automatic Classification* button to switch to the *Automatic Classification* dialog box.
13. In the *Automatic Classification* dialog box, click the *Get Min./Max. from Image* button. Then the smallest and largest value of the selected parameter that has been entered in the *Minimum* and *Maximum* fields, will be used.
    - In this way, you'll be certain that all of the objects in the image can be assigned to one of the classes that have been defined.
14. Enter the value 2 in the *Number of classes* field, and in the *Scale* field, select the *Logarithmic* entry.
    - By doing this, you have defined two size classes.

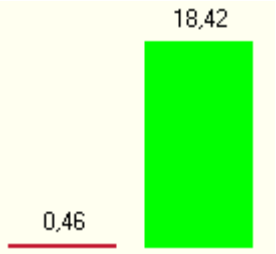
Viewing the results

15. Click *OK* and then the *Count and Measure* button, located in the *Define 'One parameter' Classification* dialog box.
    - The classes will be displayed in the image in color. The measurement parameters that have been selected for the classes will be output in the *Class Measurements* results view.



In the illustration, you can see the image with both of the size classes. The column (**1**) shows the number of large (green) and small (red) cells that was being looked for.

16. Close the *Define 'One parameter' Classification* dialog box.
    - In the *Options > Count and Measure > Classification* dialog box, the new classification is active in the list. You can now use this classification for other analyses as well.
17. Close the *Options* dialog box with *OK*.
18. Then in the *Count and Measure Results* tool window, activate the *Class Histogram* results view to have the class results displayed as a bar chart.
19. Select the *Mean (Area)* entry in the *Measurement* picklist, and the *Class* entry in the *Grouped by* picklist.
    - Now the histogram displays the mean area of the objects for each class.



In the illustration, you see the results for the object classes in the *Class Histogram* results view. The mean area ratio for the object classes is displayed as a diagram. You can clearly see that the green objects are larger than the red objects.

12.1.6. Defining macros for a batch process

Your software offers the possibility to define macros, in order to quickly and simply automate work procedures that you use repeatedly. In the batch mode, you can apply one macro to several images one after the other.

Task: You have acquired several images of different positions on the sample. You want to define a macro to automatically count and measure objects on this image.

Preparing to record a macro

1. Load all of the images that you want to analyze.
2. Select the *View > Layout > Count and Measure* command. All of the tool windows that you need for an object analysis are displayed in this layout.
3. Perform an automatic object analysis on a typical image.
   - Select the automatic threshold value setting.
   - Define a classification.
   - Specify which measurement parameters you want to use for classes and objects.
4. Close the open dialog boxes.

Creating a macro

5. Use the *View > Tool Windows > Macro Manager* command.
6. Activate an image on which no objects have yet been counted.
   - If you use an image on which an object analysis has already been performed the [Classification] command is recorded by the Macro Manager, not the [Count and Measure] command. This command leads to an error message when you apply a macro to an image on which no objects have yet been detected.
7. Click the *Create Macro* button in the tool window's toolbar. Enter a name for the new macro in the *Name* field in the *New Macro* dialog box, for example *Image analysis*. Close the dialog box with *OK*.
   - The recording of the macro starts automatically.
8. Click the *Count and Measure* button located in the *Count and Measure* tool window.

- The object analysis is now performed with the parameters that you just set.

9. In the *Count and Measure Results* tool window, activate the *Class Measurements* results view. Click the *Export to Workbook* button.
   - The results of the class measurement are exported to a workbook. The name of the workbook contains the image name by default, so that the table with the results is clearly associated with the image.
10. In the tool window's toolbar, click the *Stop Macro Running / Recording* button.
    - In the *Macro Manager* tool window's list of functions the following functions will then be listed:
      [Count and Measure]
      [Export to Workbook]
    - The macro that has been recorded will then be automatically saved. From now on you can select it in the *Macro Manager* tool window's list of macros and use it to perform a batch process.

Editing a macro

After creating a macro, you can adapt the analysis parameters to apply to other images.

1. Activate an image that you want to analyze.
2. In the *Macro Manager* tool window, activate the *Image analysis* macro.
3. In the list of functions, double click once on the icon to the right of the [Count and Measure] function.
   - The *Define Detection Options* dialog box opens. The dialog box has a tree view on the left which contains the *Segmentation*, *Detection*, and *Classification* entries.
4. Use the *Define Detection Options > Segmentation* dialog box to change the settings for the automatic setting of threshold values. You can select the *Background > Bright* option to analyze dark objects on a bright back, for example.
5. You can use the *Define Detection Options > Detection* dialog box to specify the minimum object size, for example. This dialog box contains various settings that significantly affect the number of objects that are detected and their measurement values.
6. Only with the *Count & Measure Full* solution:

   Use the *Define Detection Options > Classification* dialog box to define a new classification scheme, or to select and/or to edit a classification scheme that already exists.
7. Close the dialog box with *OK*.
   - Now when you run the macro, the newly-defined analysis parameters will be applied.

12.1.7. Performing a macro batch process for counting and measuring objects

Your software offers the possibility to define macros, in order to quickly and simply automate work procedures that you use repeatedly. In the batch mode, you can apply one macro to several images one after the other.

Task: You have acquired images at different positions on the sample. You want to automatically count and measure objects on all of the images. You want to display the results in a table sorted by image.
You have already defined a macro for this purpose.

Selecting images in the document group

1. Use the *View > Tool Windows > Documents* command to make the *Documents* tool window appear.
2. In the *Documents* tool window, select all of the images that you want to analyze. For the selection of documents, the standard MS-Windows conventions for multiple selection are valid.

Activating batch mode

3. Use the *View > Tool Windows > Macro Manager* command to make the *Macro Manager* tool window appear.
4. Select a macro with which you can count and measure objects. You can select the *Image analysis* macro that is described in the step-by-step instructions above.
   - In the *Macro Manager* tool window's list of functions the following functions should now be listed:
     [Count and Measure]
     [Export to Workbook]

5. In the toolbar of the *Macro Manager* tool window, click the *Toggle Batch Mode* button.

   - The button appears clicked, thereby showing you that the batch mode is active. You can recognize this status by the button's background color .

Starting the batch process

6. In the toolbar of the *Macro Manager* tool window, click the *Run Macro Batch* button.
   - The *Define Macro Batch* dialog box opens. This dialog box works similarly to a wizard. It guides you step-by-step through the definition of a batch process.
   - If you have already performed a macro batch process, the dialog box opens to step 3. In this case, click the *Input Definition* entry to return to step 1.

Step 1: Defining the input data

7. To start with, specify which images are to be evaluated.
   Because you want to analyze images that are currently loaded in your software, select the *Selected Documents* entry in the *Input* list.
   - The chosen images will be displayed in the *Define Macro Batch* dialog box.

8. Click the *Next >* button.

Step 2: Defining the target location

9. In the next step, you define whether and where the resulting images are to be saved. You can select the *File System* entry to save the results of the image analysis to a data storage medium, for example.
10. Click the *Browse* button to select the directory to which you want to save the results.
11. Clear the *Save images as* and *Save or export charts as* check boxes. Select the *Save or export workbooks as* check box. Select the *Excel Sheet (*.xlsx)* entry in the list.

    Now only the sheets with the results of the image analysis will be saved. They will be saved as an Excel sheet.
12. Clear the *Close documents* check box. This enables you to check the results of the image analysis on the images.
13. Click the *Next >* button.
    - The wizard's last dialog box shows you all of the settings you have made for the current batch process.

Step 3: Checking the settings for the batch process.

14. Check the settings. If you want to correct a setting, click the underlined terms. You can click the *Output definition* term if you do want to specify a different storage location for the resulting data, for example.

Performing the batch process

15. Click the *Start* button to start the batch process.
    - Your software now activates the first image and carries out the commands that have been set in the macro.
    - When the macro has been run through for the first image, the next image is activated, and this is continued until all of the chosen images have been analyzed.
    - As soon as the batch process has been started, your software opens the *Macro Batch Progress* dialog box. This dialog box gives you information about how the batch process is progressing. You can interrupt every batch process in this dialog box, or even stop it completely. Additionally, you can see a progress bar that shows you when it is expected that the batch process will be completed.
16. Close the *Macro Batch Progress* dialog box.
    - The batch process has then been completed
    - You can view all of the images that have been analyzed and their results tables in the document window.
    - The selected directory contains an Excel sheet containing the object measurements and class measurements for each image that has been analyzed.

12.2. Deep Learning

12.2.1. Overview - Deep Learning

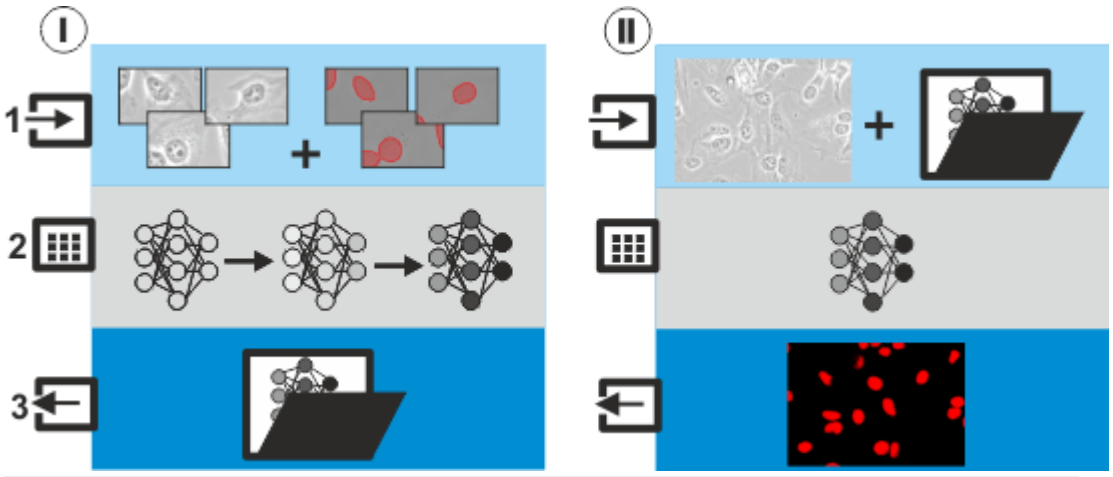
What is Deep Learning?

Many tasks require your software to detect objects in an image. One example of this is dyeing cells with fluorescing markers in order to observe and to analyze biological processes. For this to work, the cells must be automatically detected in the microscopic image acquisitions.

Deep learning can solve many of these detection tasks. Deep learning is one of the methods that machine learning uses. Deep learning uses artificial neural networks that belong to a class of algorithm that is more or less inspired by the human brain. The successful detection of cat images in an image database containing millions of animal images is the most well known example of a detection task solved with deep learning.

To be able to use deep learning, two phases are required.
In the **training phase** the neural network is trained using images of objects. These are the same objects that you later want the neural network to detect in other images, the inference images. The interesting thing is that no parameters whatsoever that characterize the objects must be specified, their size or a description of their appearance for example. All that is required is for an expert to label the objects clearly. In the cat example mentioned above, the neural network was trained using images of animals but a person was required to specify for each of the training images whether it contained a cat or not.

The second phase is the **inference phase**. The inference phase analyzes an unknown image, the inference image, using a neural network. In the cat example, a system can use a neural network to decide whether any image of an animal contains a cat or not.



The illustration shows the training phase (**I**) and the inference phase (**II**). The input data (**1**), the computation (**2**) and the output data (**3**) are shown.

The input data (**1**) in **training phase** (**I**) is microscopic images and the ground truth. In this case, the ground truths are labels indicating the objects you want to identify. In this

example, the objects you want to identify are cell nuclei. They could also be blood cells, sperm, cancerous tissue, or something else.
During the training process (**2**), your software computes appropriate parameters for the neural network. The computation runs in the background and may take a long time depending on the task and the computer being used.
After a successful training process, the objects you want to identify will be correctly detected. The training process results in a neural network with particular parameters. You save the neural network as a parameter set in a file (**3**). So the output data of the training phase is a parameter set that you can now use in your software.

In the **inference phase** (**II**) you use the saved neural network to perform an analysis on an inference image (**1**). The inference image is a microscopic image with similar objects to those in the training images. The result of the analysis using the neural network (**2**) is a probability map that informs about the likelihood for each pixel in the inference image that it belongs to one of the objects you want to identify.

Deep learning in your software

You can use the *Deep Learning* software solution to create training images, and to train neural networks. You use your own images for the training process. As a rule, you will need to train and save an individual neural network for each task that you want to solve with the help of a neural network. For the training process you require a powerful computer and an expert who can clearly label the objects you want to identify in the image.

You don't require the *Deep Learning* software solution to use a neural network in your software. You can import a neural network that was created at a different workstation.

Note: Both the training phase and the inference phase take place exclusively with your own data and on your own computer.

The difference between an analysis using a neural network, and an object analysis based on thresholds.

In the *Count and Measure* tool window, your software offers an alternative method to detect and to measure objects in images. This method of object analysis uses thresholds that are set on the distribution of color or gray intensity values in the image. This object analysis based on thresholds has a number of limitations. It requires that the objects you want to identify are distinct from the background either in color or in intensity. Objects that touch or overlap can also not be easily detected with this method. A neural network enables you to find objects that could not be found using a standard object analysis based on thresholds.

General process flow of an analysis using a neural network

The following steps are required to train and apply a neural network using your software.

Perform the training phase

Step 1: Acquiring training images

Acquire the training images. Use similar acquisition conditions (exposure, magnification) for

the training images to those you will later use to acquire the inference images that you want to analyze with the neural network.

Step 2: Creating training labels

On the training images, define the objects that you want the neural network to detect. You can define the objects either automatically or manually. If you want to define the objects automatically, use the *Count and Measure* tool window to perform an automatic object analysis using thresholds. If you want to define objects manually, use the *Training Labels* tool window.

Step 3: Training and saving a neural network

Select the training configuration for the neural network and a training duration. Start the training process.

Follow the progress of the training process. During the training process, you can check the results on validation images at different points in time.

When your training process has been successful, save a new neural network as a parameter set.

Use the *Deep Learning* layout to train and save a neural network.

Performing the inference phase

In your software, there are several ways of performing an analysis using a saved neural network.

Option 1: Performing an object analysis on a probability map

This option first performs an analysis using a neural network. Then an object analysis is performed on the probability map.

To perform the analysis using a neural network, use the *Process > Deep Learning > Neural Network Processing* command. The neural network will identifies object in the inference image. The result is a probability map. For each pixel of the inference image, the probability map indicates the likelihood that the pixel belongs to an object.

Then use the *Probability Layer Segmentation* software function in the *Count and Measure* tool window to perform an object analysis on the probability map. This will detect and measure the objects in the probability map.

Option 2: Performing an object analysis using a neural network

This option combines the analysis using a neural network, and the object analysis into one step.

Use the *Neural network Segmentation* software function in the *Count and Measure* tool window to perform an analysis using a neural network on the inference image. The neural network will identify objects in the inference image. Then the objects will then be detected and measured immediately. You can view the measurement results in the *Count and Measure Results* tool window.

Option 3: Applying the neural network in the live image

Use the *Live AI* tool window to analyze the live image using a neural network. This enables you to detect and count the required objects in the live-image.

Software and hardware requirements

Software requirements

You must purchase the *Deep Learning* software solution to train a neural network.

Which graphics cards are supported?

A large amount of data must be processed to train a neural network. This demands a lot of the PC's hardware equipment and requires a fast graphics card. To be suitable, a graphics card must support CUDA technology.

The two graphics cards listed below were successfully tested. However, due to technical progress, this list can change frequently. Contact your Olympus sales representative if you have any questions about suitable graphics cards.

NVIDIA Quadro P4000
NVIDIA Quadro RTX 4000

What are training images?

The neural networks that you train with your software should be able to identify very specific types of objects in an image. The software requires training images to perform a training. The number of training images required to train the neural network very much depends on the task.

Requirements for the training images

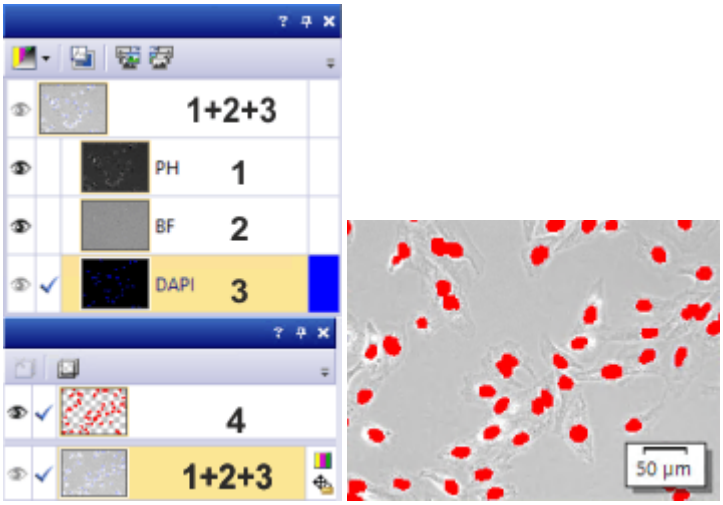
The training images must meet the following requirements:

Images with training labels	All training images must contain training labels. The training labels form the ground truth for the training of the neural network. You can automatically create the training labels using an object analysis. However, this will only be successful if the objects to be found have a color or intensity that is distinct from the background. Use the *Count and Measure* tool window to perform an automatic object analysis. If the training images aren't suitable for an automatic object analysis, you can create the required training labels manually. Use the *Training Labels* tool window to manually draw the training labels. The training labels are displayed on their own image layer. This image layer is called *User Labels*.
Training label classes	With your software, you can create neural networks that search for different classes of objects simultaneously in images. The training images for these types of neural networks will then contain different training label classes. A separate class is defined for each object type.

Image size	The minimum size for training images is 512x512 pixels. The training images don't have to be of the same size. As long as a training image has the required size, you may use it. If you are using a very large image with very many objects as a training image, a single image may suffice to train the neural network. Please note that this requirement only applies for the training images. You can later also apply the neural network to images that have a different image size.
XY-calibration	All of the training images must have approximately the same calibration. If you acquired all the training images with the same objective magnification, this requirement will be fulfilled.
Image format	Training images must be in one of the following image file formats: VSI, TIF, TIFF, BTF.

Examples of suitable training images

Let's assume you want the neural network to detect cell nuclei in a phase contrast image or in a brightfield image. In this case dye the cell nuclei with a suitable fluorochrome, DAPI for example. Acquire the fluorescence images of the sample together with a brightfield image and a phase contrast image. In the fluorescence image, detect the cell nuclei on the DAPI fluorescence channel. You won't need the training images an more when you apply the neural network later. Using the neural network you will be able to detect the cell nuclei directly on the phase contrast images or the brightfield images.



At the top of the illustration on the **left** you can see the *Dimension Selector* tool window with the individual channels in the training image. Under that you can see the *Layers* tool window. The *Layers* tool window contains two image layers. Image layer (**4**) is the *Detected Objects* layer, the one that contains the cell nuclei that were detected. The second image layer is the training image with the three channels (**1+2+3**).
The illustration on the **right** shows how the training image looks in the image window after the object analysis is finished. The detected cell nuclei are colored red and can be found on the *Detected Objects* layer over the training image. In this example the red color of the detected objects covers the blue DAPI dye.

The resulting probability map

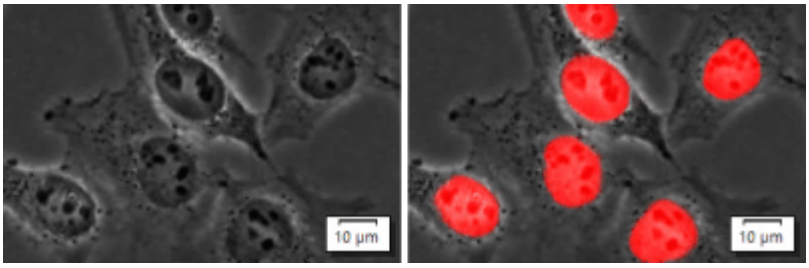
A deep learning analysis produces a probability map. For each pixel of the inference image, the probability map indicates the likelihood that the pixel belongs to an object. You can define the color that the probability map will use in the *Training Labels* tool window. The probability map isn't a binary image. It has varying intensity values. The intensity corresponds to the probability that an object that was predicted is actually present in the inference image. If an object on the probability map is only very lightly colored, there is a small probability that an object actually exists at that position. If the object is heavily colored, there is a high probability that the prediction is accurate.

The probability map is a separate image layer that is superimposed on the inference image. Use the *Layers* tool window to show or hide the probability map. You can also extract the probability layer.

When the neural network was trained using several training label classes, a separate probability map is calculated for all defined training label classes. In this case, use the *Dimension Selector* tool window to show or hide the probability maps for the individual training label classes.

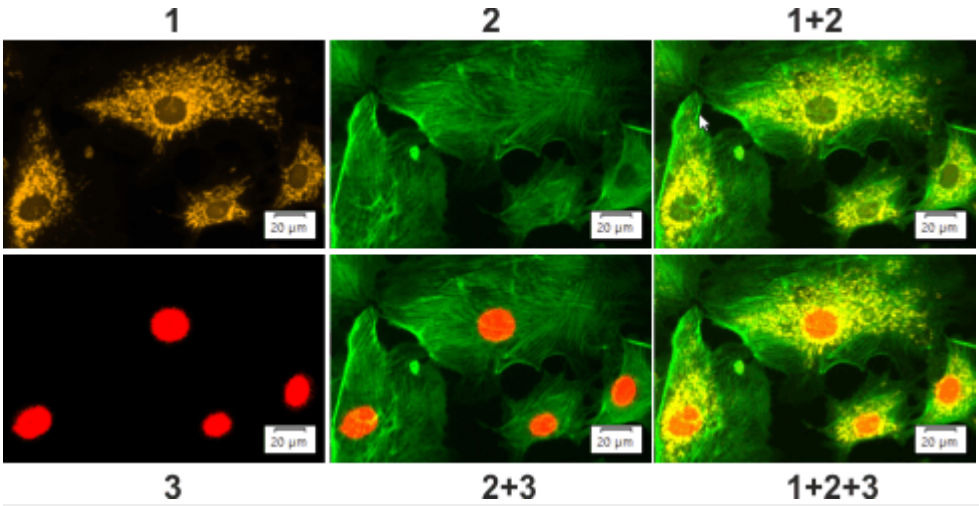
Examples for the resulting images

Example 1: The neural network recognizes cell nuclei on a phase contrast image.



The left image shows the phase contrast image on which the cell nuclei are to be counted. The right image shows the image resulting from an analysis using a neural network. The probability map is superimposed on the phase contrast image. The cell nuclei were recognized correctly and are colored by the probability map. In this case, the probability map uses the color red.

Example 2: The neural network recognizes cell nuclei on a multi-channel fluorescence image with two fluorescence channels.



The input image consists of the two fluorescence channels (**1**) and (**2**). Image (**1+2**) shows the superimposition of the two fluorescence channels.
The neural network computes a probability map (**3**). The probability map indicates the likelihood that a cell nucleus is located at a certain position in the inference image. You can superimpose the probability map on an individual fluorescence channel (**2+3**).
Image (**1+2+3**) shows the superimposition of all image layers and channels.

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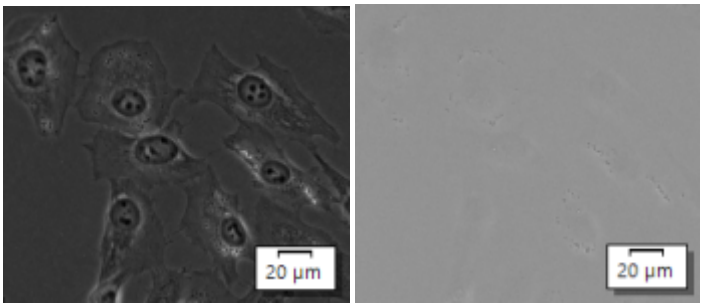
12.2.2. Analyzing images using deep learning

Example: Lets say you want to determine the number of cell nuclei on a phase contrast image or on a brightfield image. In both phase contrast images as well as in brightfield images the cell nuclei are the same color as the background, making it hard to detect the cell nuclei automatically. This is why it's usual to dye the cell nuclei with a fluorochrome and to acquire a fluorescence image of the sample.
You can use the *Deep Learning* software solution to train a neural network to find the cell nuclei in a phase contrast image or a brightfield image without having to make a fluorescence acquisition. This way you can avoid the laborious preparation of the fluorescence sample and the fluorescence acquisition.



The illustration on the left shows the phase contrast image and the illustration on the right shows the brightfield image. In both images the cell nuclei are the same color as the background, making it hard to detect the cell nuclei automatically.

Example data for deep learning

After you have installed your software you can install various data that might be helpful when familiarizing yourself with *Deep Learning*. There are training images, inference images, and a neural network that has already been trained.

You can install the example data as soon as you've installed the software, or at any later point in time. To do so, insert the DVD that contains the software into the DVD drive. If the installation wizard starts, browse to the directory that contains the example data and install them.

Note: If your computer doesn't have the required graphics card or if you don't want to label the objects manually on the training images, you can use the neural network provided.

Step 1: Acquiring training images

Example: Acquire a multi-channel image of the sample that contains a phase contrast image, a brightfield image, and a fluorescence image.

Note: This set of step-by-step instructions is just one example of how to acquire training images. You can also acquire training images in other ways or use existing images.

Prerequisites:
Your microscope is equipped to acquire fluorescence images.
The system has been correctly configured.

Preparing for the acquisition of training images

1. In a sample, dye the cell nuclei with a fluorescence color, DAPI for example.
2. Define an observation method for phase contrast acquisitions *PH*, for brightfield acquisitions *BF* and for the fluorescence channel *DAPI*.
3. Place the sample under the microscope.
4. Select the microscope settings you want. For example, choose a suitable magnification and illumination intensity.

Define the name and storage location for the training images

1. Use the *View > Tool Windows > Process Manager* command to make the *Process Manager* tool window appear.

2. Click the *Acquisition Settings* button, located on the *Process Manager* tool window's toolbar.
3. Select the *Saving > Process/Experiment* entry in the tree view.

   Select the *File system* entry in the *Automatic save > Destination* list to automatically save the training images you have acquired.

   Click the [*...*] button next to the *Path* field to change the directory into which the training images are saved.

Note: Your software must have access to the training images for the whole duration of the training. So use a directory where access is guaranteed.

- Images that you acquire in the *Process Manager* tool window are automatically saved in the VSI image format.

4. Select the *Document Name > Process/Experiment* entry in the tree view.

   Click the *All Options* button.

   Specify a name for the training images. For example, you can combine the *Experiment Name*, *Separator* and *Counter* placeholders to create the name.

   Close the *All Options* dialog box with *OK*.
5. Close the *Acquisition Settings* dialog box with *OK*.

Defining the acquisition process for the acquisition of a multi-channel image

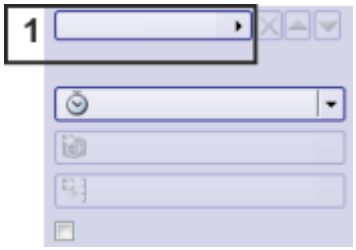
Define the acquisition process in the *Process Manager* tool window.

1. Assign a name to your training images in the *Experiment name* field. You can find the field in the first group in the *Process Manager* tool window.

   You could call them *Training Image PH BF DAPI* for example.
2. Select the *Automatic Processes* option.

3. Use the *Multi Channel* acquisition process to acquire multi-channel images of the sample. To do so, click the *Multi Channel* button.

4. Click the *Add Channel* button (**1**).



   - A context menu opens. The context menu lists all of the observation methods that are currently defined.

5. Select the *PH*, *BF* and *DAPI* channels in sequence.

An acquisition process has been defined for the *PH*, *BF* and *DAPI* channels in the *Process Manager* tool window.

6. Click the small plus sign to the left of the channels to view the current settings for the channels.

   If necessary, clear the *Transmission overlay* check box for the *PH* or *BF* channel. A transmitted light image becomes an image layer on top of the multi-channel image. In this example, the acquired image should not contain such an image layer. All acquired images should be combined to a multi-channel image.

7. Switch to the live mode. To do this, you can click the *Live* button, located in the *Process Manager* tool window's toolbar.

   Specify the appropriate exposure time for the individual channels. To do so, select the individual channels. Click the *Auto Exposure* button to automatically determine and adopt the exposure time for the selected channel. You can find this button just below the table with the channels.

8. Bring the image into focus.

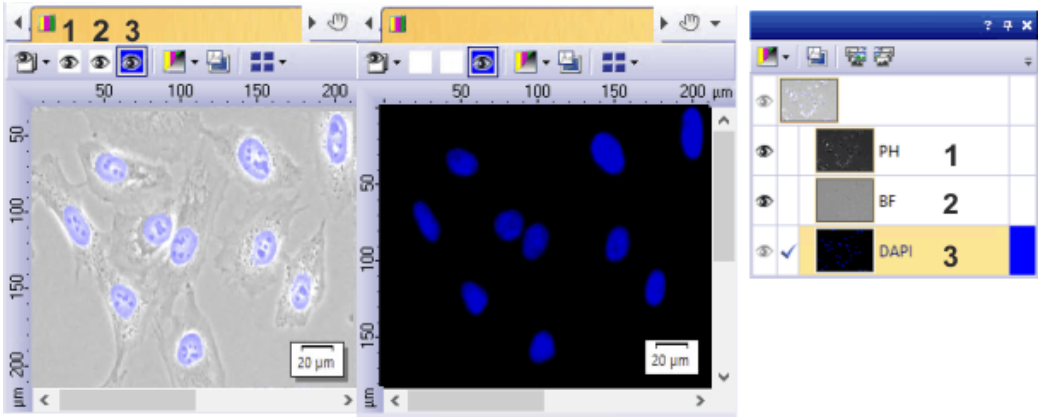
   Click the *Read Z-offset* button to adopt the current Z-position of your microscope stage for the selected color channel.

9. Finish the live mode.
10. Select the *Use Z-offset* check box.

Acquiring and loading training images

1. In the *Process Manager* tool window, click the *Start* button.
   - The acquisition begins immediately.
   - You can find the saved image in the directory that you specified for the training images. The training images are called *Training_PH_BF_DAPI_01.vsi*, *Training_PH_BF_DAPI_02.vsi* and so on.
2. Go to a different position on the sample and acquire additional training images.

   The number of training images required to train the neural network very much depends on your use case. If you want to check the results during the training process, acquire enough images so that one of the images is left over to be used as a validation image. This will give you a visual way of checking the results of the training while it is in progress.
3. Load the training images in your software.
4. Use the *View > Tool Windows > Dimension Selector* command.
5. Take a look at the training images.
   - The training images are multi-channel images with the 3 channels *PH*, *BF* and *DAPI*.
   - Each channel is listed in the *Dimension Selector* tool window.
6. Continue the deep learning object analysis. In the next step, you will define the training labels on the *DAPI* channel.



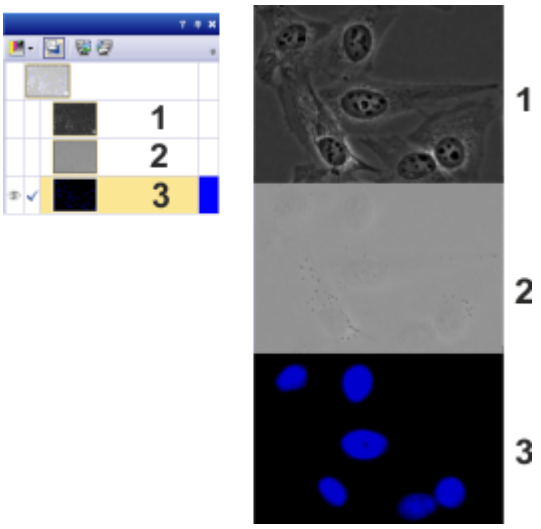
In the image window (**left**), you can see all of the channels superimposed on each other. You can use the buttons (**1**), (**2**) and (**3**) in the image window's navigation bar to show and hide individual channels. When all of the images are superimposed, the cell nuclei are light blue. This is because the *DAPI* channel (which has been dyed blue) is showing through the phase contrast image and the brightfield image, both of which have poor contrast.
The illustration in the **middle** displays only the *DAPI* channel with the cell nuclei. The *PH* and *BF* channels are not shown. You will define the training labels on the *DAPI* channel in the next step.
On the **right** you can see the training image in the *Dimension Selector* tool window. In this tool window. you can see that the image is composed of three channels.

Step 2: Creating training labels

Example: Create the training labels. To do this, on the training images define the objects that you want the neural network to detect.
In this example, you employ the automatic object analysis using thresholds to detect the cell nuclei on the *DAPI* channel. The *DAPI* fluorescence channel is particularly well suited for an automatic object analysis using thresholds because the cell nuclei are so clearly a different color from the background.

Note: This set of step-by-step instructions is just one example of how to automatically create training labels. You can also draw the training labels manually. To do so, use the *Training Labels* tool window.

Prerequisite: Your software has the *Count & Measure Full* solution.



The training images have three channels; *PH* (**1**), *BF* (**2**) and *DAPI* (**3**). You want to detect the nuclei on the *DAPI* channel.

Preparations

1. Display the following tool windows: *Count and Measure*, *Count and Measure Results*, *Dimension Selector*, and *Layers*.

   To do this, select the tool windows from the *View > Tool Windows* menu.
2. Load the training images in your software.
   - During the installation of your software some sample images have been installed, too. You can follow this step-by-step instructions using the *Training Image_PH BF DAPI_01.vsi*-*Training_PH BF DAPI_10.vsi* example images.
3. If you don't want the training images to change, save them in a different directory.
4. Activate the first training image.

Setting options

5. Open the *Options* dialog box by clicking the *Count and Measure Options* button, located in the *Count and Measure* tool window.
6. Select the *Count and Measure > General* entry in the tree view.

In the *Segmentation and detection target* group, select the *Selected frames and channels* option. Now, you can perform the object analysis for the individual channels in a multi-channel image.

7. Select the *Count and Measure > Classification* entry in the tree view.

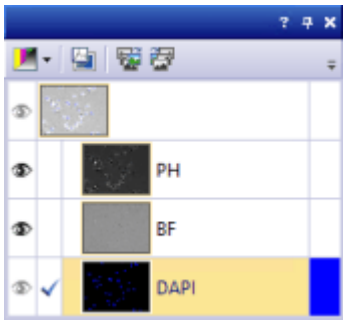
   For this example, use the *Phase* predefined classification. Using this classification, all objects belonging to one phase form their own class. If you later define only one phase for the *DAPI* channel, all of the nuclei will form a single object class.

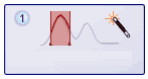
Note: The *Phase* classification is suitable for this example. For your application you can select more than one class for your objects. You can use your classes to distinguish between large and small objects for example. Or you can subdivide the objects into one class of oval objects and one class of round objects.

8. Close the *Options* dialog box with *OK*.

Setting threshold values

9. Activate the *DAPI* channel in the first training image. To do so, click the *DAPI* button in the *Dimension Selector* tool window.
   - In the *Dimension Selector* tool window, the active channel is highlighted and identified by a check mark.





10. In the *Count and Measure* tool window, click the *Automatic Threshold* button to open the *Automatic Threshold* dialog box.
    - The three channels are automatically displayed in the *Channel* group, under their names.
11. Select the *DAPI* channel in the *Automatic Threshold* dialog box.

    Check whether the objects have been correctly detected. Should the objects not have been correctly recognized, go to the *Background* group and enter whether the background is bright or dark. For this example, select the *Background > Dark* option since the image has bright objects against a dark background.
    - Only the selected channel is shown in the image window.
    - For an object analysis using thresholds, the intensity distribution of the image is divided into different phases. In this example, the cell nuclei form a phase of bright objects on a dark background.

      The cell nuclei are given the same color as the phase.
12. Make sure that only one phase has been defined. Should more than one phase have been defined from an earlier analysis, delete the superfluous phases by clicking the *Remove Phase* button.

Assign a name to the phase. To do so, double click in the *Phase Name* cell and enter a name, *Cell nuclei* for example.

Select a color for the phase. You can select red, for example. This will later enable you to easily distinguish the training labels from the blue nuclei.

Note: Don't select a color that is already being used in any of the image's other channels. To see the assigned phase color for a channel, select the channel in the *Automatic Threshold* dialog box located in the *Channel* group.

- In the image window, the cell nuclei are given the same color as the phase.

The illustration shows the image window with the training image. The *Automatic Threshold* dialog box is also displayed.

Performing an automatic object analysis using thresholds

13. To carry out the object analysis, click the *Count and Measure* button in the *Automatic Threshold* dialog box.
    - The automatic object analysis is only carried out on the *DAPI* channel. The cell nuclei are now the same color as the phase.
    - The *Automatic Threshold* dialog box is closed.
    - The number of objects found is displayed in the *Object Count* group in the *Count and Measure* tool window.
    - A new image layer is created for the objects that have been detected. This image layer is called *Detected Objects*. Use the *Layers* tool window to show and hide these image layers.

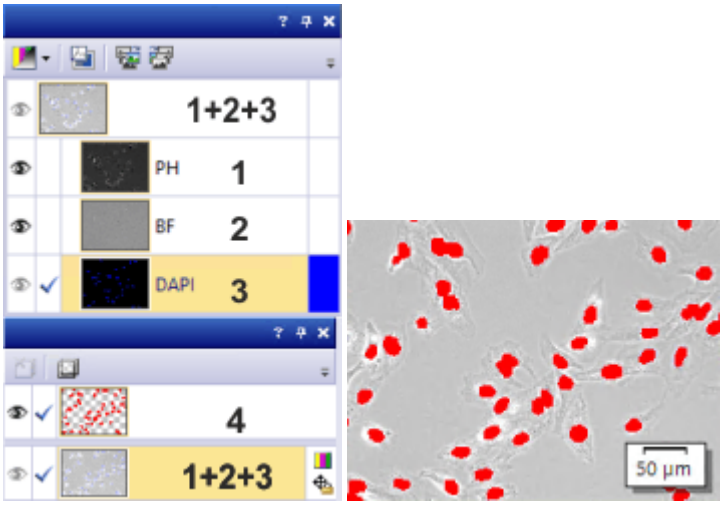
Note: For neural network training, the training labels must be in the *User Labels* layer. When the training is set up, the detected objects are automatically copied from the *Detected Objects* image layer to the *User Labels* image layer.

14. Save the training image with the detected nuclei.

Viewing the results

15. Use the *Layers* tool window to show and hide the *Detected Objects* image layer.
16. In the *Count and Measure Results* tool window, select the *Class Measurements* results view.

- Your software automatically creates an object class for each channel in the image. So three object classes are listed in the *Class Measurements* results view. The **1** (green by default) and **2** (yellow by default) object classes belong to the *PH* and *BF* channels. These object classes don't contain any objects. The *Cell nuclei* (red) object class is the object class with the cell nuclei. This is the one that is important for the training.



At the top of the illustration on the **left** you can see the *Dimension Selector* tool window with the individual channels in the training image. Under that you can see the *Layers* tool window. The *Layers* tool window contains two image layers. Image layer (**4**) is the *Detected Objects* layer, the one that contains the cell nuclei that were detected. The second image layer is the training image with the three channels (**1+2+3**).
The illustration on the **right** shows how the training image looks in the image window after the object analysis is finished. The cell nuclei now appear red. This is because the *Detected Objects* layer is on top of the training image.

Performing an object analysis on the other training images

1. Activate the next training image.
2. Activate the *DAPI* channel in the *Dimension Selector* tool window.
3. Click the *Count and Measure* button located in the *Count and Measure* tool window.
   - All of the settings that have been specified for the automatic object analysis are retained.
4. Save the training image.
5. Perform the object analysis on all of the training images.
6. Continue the deep learning object analysis. In the next step, you will train and save the neural network.

Note: If you have a large number of images to analyze, you can define a macro to apply a batch process to all of the training images that have been saved. To do so, use the *Macro Manager* tool window.

Step 3: Training and saving a neural network

Example: Use the training images from step 2 to train a neural network. Let's assume you want the neural network to find cell nuclei on a phase contrast image. Then train another neural network to find cell nuclei on brightfield images.

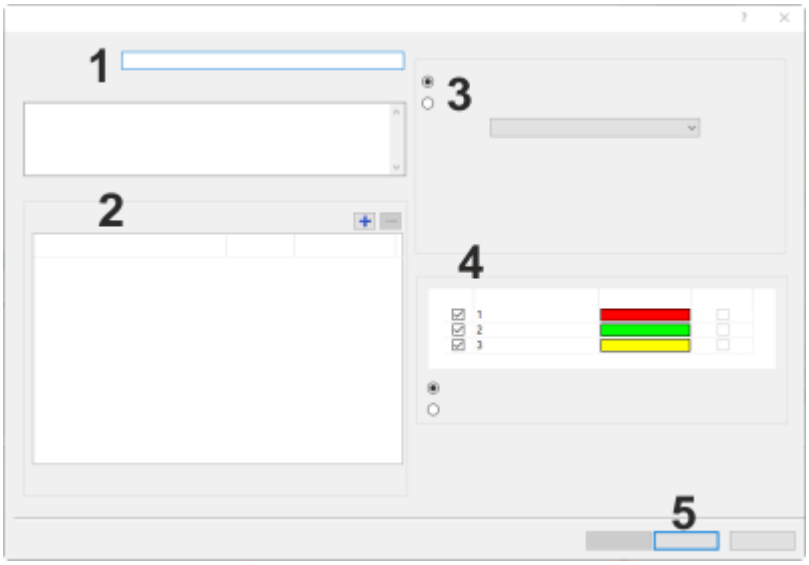
Starting a new training process

1. Switch to the *Deep Learning* layout. To do this, click the *Deep Learning* tab at the top right of the user interface.
   - The *Deep Learning* layout has been designed especially for training neural networks. In contrast to other layouts in your software, the *Deep Learning* layout always has the same structure. It also fills the whole user interface.
2. If you don't want the training images to change, save them in a different directory.
3. For neural network training, the training labels must be in the *User Labels* layer. If you performed an automatic object analysis using thresholds to create the training labels, the training labels will be in the *Detected Objects* image layer. When the training is set up, the detected objects are automatically copied from the *Detected Objects* image layer to the *User Labels* image layer. This changes the training images. The file size of the training images increases.

3. Click the *New Training* button. You can find the button at the top left of the *Deep Learning* layout.
   - The *New Training: Input and Output* dialog box opens.

Specifying the input and output for the training

Make the necessary settings in the *New Training: Input and Output* dialog box.



1. In the *Name* field (**1**), enter a descriptive name for the neural network that you want to create. For this example, you can name the training *PH_nuclei*.

   In the *Description* field, enter a good description of the new neural network.
   - Your software continually checks the settings in the *New Training* dialog box. If a setting has not yet been made or if a settings is incorrect, a message appears

at the bottom right of the dialog box.

Before you have selected the images, a message appears stating that no input images have been specified yet. This message disappears as soon as you add the training images.

2. Click the [+] button in the *Images* group (**3**).

   Navigate to the directory in which your training images are saved and select the training images.

   Click the *Open* button to load the training images.

   - Your software recognizes that the training images contain detected objects and asks you whether you want to convert the detected objects into training labels. This automatically copies the *Detected Objects* image layer to the *User Labels* layer.

3. Confirm the message with *Yes*.

   - The detected objects are copied from the *Detected Objects* image layer to the *User Labels* image layer.
   - The imported training images are displayed in the *Images* group.
   - Your software recognizes the three channels, *PH*, *BF* and *DAPI*, that compose the training images. The channels are displayed next to the name and the size of the training images.
   - The *Training label classes* group (**4**) lists the training label classes that have been defined for the training images. When the training images are imported, the object classes are automatically converted into training label classes.

     With your software, you can create neural networks that search for different classes of objects simultaneously in images. A separate class is defined for each object type.

     In this example there is only one single relevant object class, the *Cell nuclei* training label class. The training label classes are listed anyway, because the training images have the multi-channel image image type. During an automatic object analysis your software automatically creates an object class for each channel in the image. Each object class is converted into a training label class. The *1* (green) and *2* (yellow) training label classes belong to the *PH* and *BF* channels. These classes don't contain any objects. The *Cell nuclei* (red) training label class is the class with the cell nuclei. This is the one that is important for the training.

4. In the *Training label classes* group (**4**) clear the check boxes to the left of classes *1* and *2*. These classes aren't relevant to the training.

   In this example, all of the objects that you want to analyze with the neural network belong to the same class. That means that for this particular example it's not important whether you select the *multi-label classification* option or the *multiclass classification* option.

5. For neural network training you have to select at least one channel in the training images. During the training, the neural network only tries to find training labels on the selected input channels (in this example cell nuclei).

You want to train a neural network that finds cell nuclei in phase contrast images. The correct input channel for this neural network must therefore be the *PH* channel. In the *Input channels > Name* list (**3**), select the *PH* entry.

6. Click the *Next* button (**5**) to select the training configuration for the neural network.
   - The *New Trainings: Parameters* dialog box opens.

Specifying parameters for the neural network

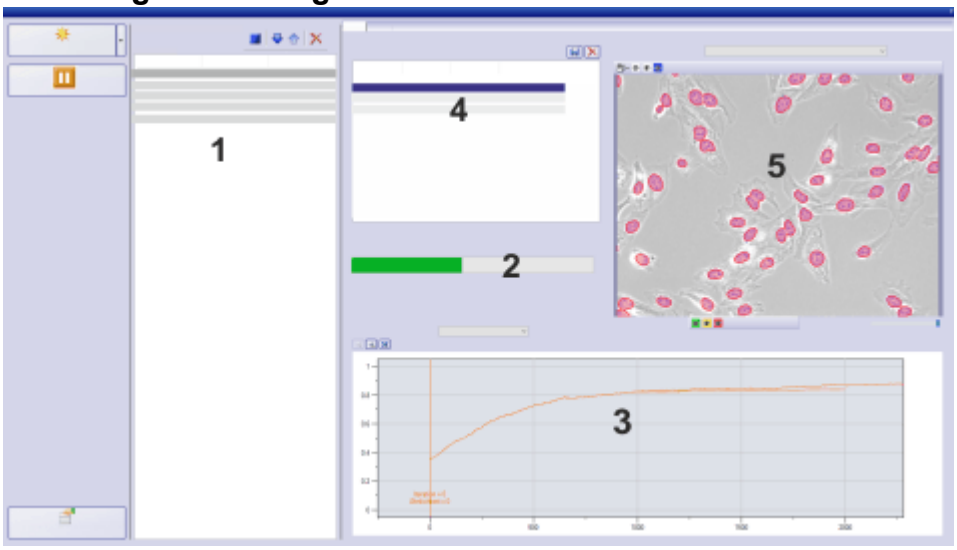
1. The *Deep Learning* software solution uses pre-configured training configurations for the neural network. From the *Available training configurations* list, select the training configuration that you want to use for the training process. To the right of the selected training configuration, you can find a detailed technical description.

   Which parameter set delivers the best results depends strongly on the task. The *Standard Network* parameter set is suggested for most applications.

   For this example, select the *Standard Network* parameter set.
2. The cell nuclei are still easily recognizable in the phase contrast images. In this example, you can reduce the duration of the training process. In the *Training duration* list, select the *Iteration limit* entry.

   Enter the required number of iterations in the field to the right of the *Training duration* list. For this particular example, select 5000 iterations.
3. Click the *Start* button to start the training process.
   - The *New Training* dialog box closes.
   - You can follow the progress of the training in the *Deep Learning* layout.

Following the training of the neural network



The *Deep Learning* layout will look similar to this while the training is in progress.

1. You can follow the progress of the training in the *Deep Learning* layout.
   - The trainings that your have defined or already performed are listed in the training list. When a new training begins, it is inserted into the top position in the training list (**1**). The training that is in progress has the *Running* status.

- The progress bar (**2**) shows when the training is expected to finish. The progress bar tells you the total number of iterations that have already been performed, and how many are yet to come. The time remaining in the training process is displayed next to it.
- Your software provides several quality indicators. These enable you to check the quality of the neural network. The *Similarity* quality indicator is displayed by default in the diagram (**3**). The diagram is refreshed constantly while the training is in progress.

  The *Similarity* value is between 0 and 1. The closer the value is to 1, the better the prediction of the neural network is. In this example, the curve climbs and approaches the value of 1. This curve shows that the neural network that is being trained is finding the cell nuclei increasingly well.
- The neural network consists of a parameter set. This parameter set is varied during the training and adapted to the training images. Your software saves the current parameter set at regular intervals and uses it to create checkpoints. You can use these to check the quality of the neural network. The checkpoints are listed in the *Available checkpoints* (**4**) list.

  In this example, checkpoint 1 is created after 1000 iterations. This checkpoint reflects the development of the neural network's parameter set at 1000 iterations.
- The validation image (**5**) shows the result that belongs to the selected checkpoint. This means that, at checkpoint 1, the neural network analyzes the validation image with the parameters that were calculated after 1000 iterations.

  For the first checkpoint in the list, the calculation has not yet started. No neural network has yet been calculated. The validation image shows one of the training images without a probability map. For each pixel, the probability map indicates the degree of probability that the pixel belongs to a class.

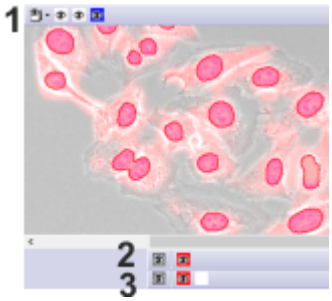
2. Take a look at the probability map for one of the checkpoints that has already been computed. You can select checkpoint 3 in the *Available checkpoints* list for example.
   - The preview window in the *Deep Learning* layout displays all of the image layers in the validation image superimposed on top of each other. Use the buttons over and under the validation image to show and hide the different image layers.

     The buttons (**1**) above the validation image show the channels that compose the training images. In this example, the training image contains the *PH*, *BF*, and *DAPI* channels.

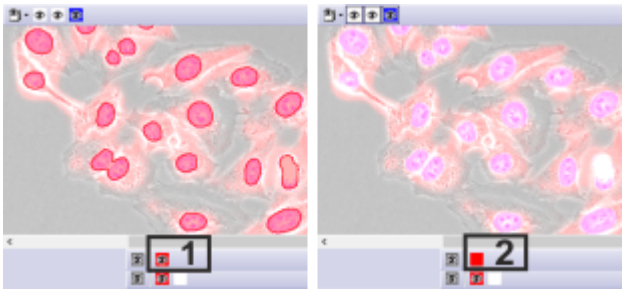
     The *Training label classes* buttons (**2**) under the validation image correspond to the training label classes that have been defined. In this example, only one training label class is active for the training job. The red training label class contains the training labels for the cell nuclei.

     The *Probability* buttons (**3**) correspond to the probability maps for the individual training label classes. A separate probability map is created for each training label class. Additionally, a probability map is always created for the

background.

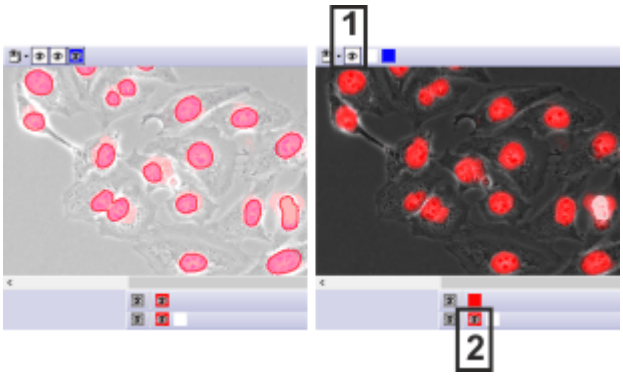


3. Click on one of the buttons to show or hide the corresponding image layer. You can repeatedly click on the red *Training label classes* button under the validation image.
   - The training labels appear and disappear. This allows you to judge whether the neural network belonging to the selected checkpoint is finding the cell nuclei as wanted.



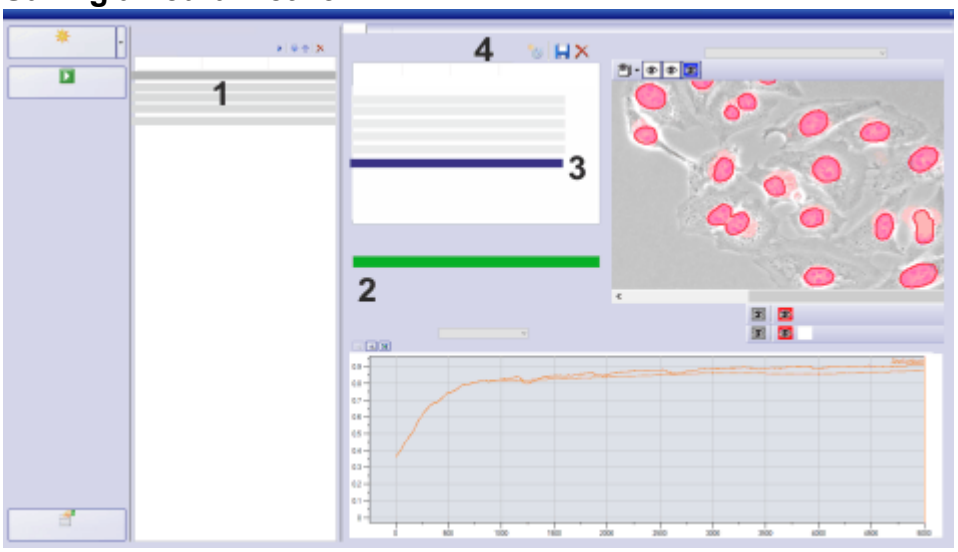
On the left, the image layer with the training labels is shown (**1**). On the right it is hidden (**2**). The checkpoint was saved after 200 iterations. The red areas in the image on the right display the probability map. After as few iterations as these, the probability map doesn't correspond with the training labels very well yet.

4. You can now also hide the *BF* and *DAPI* channels. To do this, click the buttons that belong to these channels.
   - Now only the probability map for the cell nuclei and the phase contrast image are shown (superimposed on each other). This way of displaying the image makes it particularly easy for you to judge whether the neural network fulfills it's purpose.



On the left, all of the image layers are shown. On the right, only the *PH* channel (**1**) and the probability map (**2**) are shown. The checkpoint was saved after 1000 iterations. The neural network after 1000 iterations finds the cell nuclei significantly better than it did after 200 iterations.

Saving a neural network



After the training is finished the *Deep Learning* layout will look similar to this.

1. Wait until the neural network training is finished.

Note: You can continue to use your software while a training process is running. You can also define additional training processes. The trainings will then automatically be performed one after the other.

- After the training is finished, its status changes from *Running* to *Done* (**1**).
- The progress bar (**2**) shows you that the training is finished.

2. Select the checkpoint (**3**) at which the similarity is the highest. As a rule, it will be the last checkpoint.
3. Click the *Save Neural Network* button (**4**). You can find the button above the *Available checkpoints* list.
   - The *Save Neural Network As* dialog box opens.
4. Enter a descriptive name for the neural network in the *Name* field. For this example, use the name *NN_Nuclei_PH*.

   Use the *Description* field to describe the particular application and the training images being used.

   If you want other users of your software to be able to use the neural network, select the *Public* option.

   Click the *Save* button.
5. You can now use the *NN_Nuclei_PH* neural network to analyze cell nuclei on phase contrast images.

Starting a new training to analyze brightfield images

Example: The following step-by-step instructions are only required if you want to analyze brightfield images in addition to phase contrast images. To do this, train and save a new neural network.

1. In the training list, select the training that you just defined.
2. Click the small arrow next to the *New Training* button to open a small menu. Use the *New Training from Selected* command to define and start another training based on an these settings.
   - The *New Training from Selected: Input and Output* dialog box opens. All of the settings for the training that has already been performed will be adopted.
3. Enter a new name for the training job in the *Name* field. You could call it *NN_Nuclei_BF* for example.
4. You want to train a neural network that finds cell nuclei on brightfield images, not phase contrast images. The correct input channel for this neural network must therefore be the *BF* channel. In the *Input channels > Name* list, select the *BF* entry.
5. Click the *Next* button.
6. The cell nuclei are significantly more difficult to find on the brightfield images. For this reason, select the *No limit* entry in the *Training duration* list.
7. Follow the progress of the training process.
8. If you are happy with the results, stop the training job.

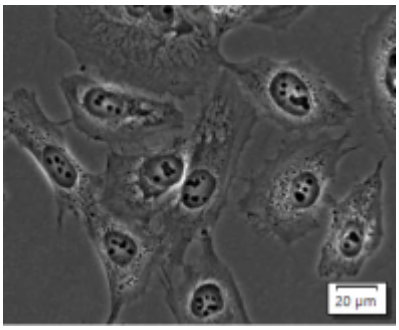
   To do so, click the *Stop Training* button. You can find the button in the *Deep Learning* layout above the list of training jobs.
   - A message appears asking whether you would like to write a checkpoint.
9. Confirm the message with *Yes*. Wait until the checkpoint appears in the *Available checkpoints* list.
10. Select the last checkpoint and save the neural network under the name *NN_Nuclei_BF*.

    You can now use this neural network to analyze cell nuclei on brightfield images.

Step 4: Applying a neural network

Example: Use the *NN_Nuclei_PH* neural network you created in step 3 to find cell nuclei on phase contrast images.

Note: You don't require fluorescence samples when you apply the neural network.



The images show a phase contrast image that you can analyze using the calculated neural network *NN_Nuclei_PH*.

Acquiring images

Note: You can of course apply the neural network to existing images. In this case, load the image that you want to analyze.
During the installation of your software some sample images have been installed, too. You can follow these step-by-step instructions using the example images in the *DeepLearning > Inference image* directory.

1. Switch to the *Acquisition* layout. To do this, you can use the *View > Layout > Acquisition* command.
2. Select acquisition conditions that are as similar as possible to those used to acquire the training images. For example, select the same objective magnification and similar exposure conditions.

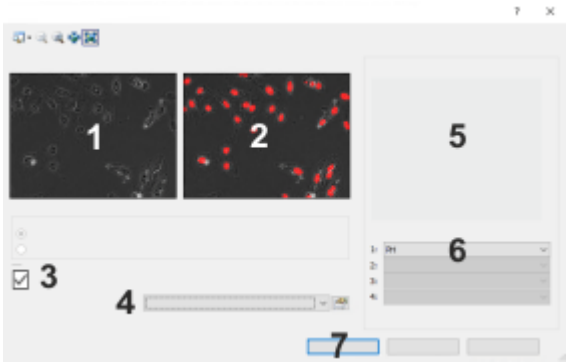
   Only images that have the same input channels as the training images can be analyzed. Therefore use the *PH* observation method that you defined for the acquisition of the training images.

3. Acquire an image that you want to analyze. To do this, you can click the *Snapshot* button in the *Camera Control* tool window.
   - The acquired image is shown in your software's image window.

Applying a neural network

1. Use the *Process > Deep Learning > Neural Network Processing* command.
   - The *Neural Network Processing* dialog box opens.
   - The active image is displayed in the left of the preview area (**1**).
   - Your software starts the analysis as soon as a suitable neural network has been selected. It can start as soon as you open the dialog box. As soon as the analysis is finished, the resulting image is displayed in the preview area (**2**).

     If you are analyzing very large images, you can zoom in to them in the preview area. The preview area then displays a smaller section of the image and the resulting image can be computed more quickly. To do this, use the button above the preview area.

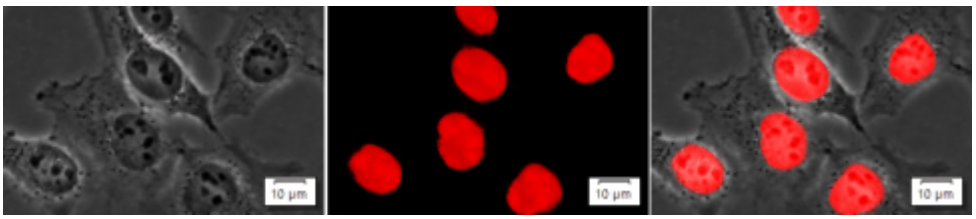


2. Select the *Create new document as output* check box (**3**). Now the data in the inference image will remain unchanged.
3. In the *Neural network* list (**4**), select the required neural network for the analysis. For this example, load the *NN_Nuclei_PH* neural network.

   In the *Network properties and settings* group (**5**), you can find the description of the neural network that you entered when you saved the neural network. Use the description to identify a suitable neural network.

   - If the selected neural network doesn't suit the active image, a message appears under the *Neural network* list in the dialog box.
   - Your software starts the analysis as soon as a suitable neural network has been selected.
4. In the *Input channel assignment* list (**6**), select the channel of the input image that you want to analyze.

   In this example, the input images are only from the *PH* channel. That's why the list only contains one entry.
5. Click the *OK* button to close the *Neural Network Processing* dialog box and to open the resulting image in the image window. Pay attention to the progress bar located in the status bar.
   - A deep learning analysis results in a multi-channel image that contains the *Probability map* image layer in addition to the input image.
   - The resulting image is shown in the image window.



The illustration shows the input image at the left, the probability map in the middle, and the probability map superimposed on the input image at the right.

Viewing the resulting image

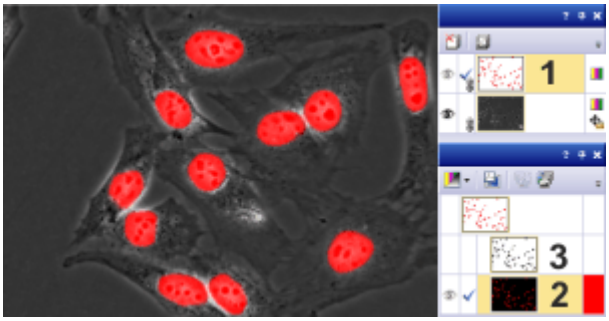
Take a look at the image resulting from the deep learning object analysis.

1. Display the *Layers* and *Dimension Selector* tool windows. Both of these tool windows are shown by default in the *Processing* layout. Look at the resulting image in the *Layers* tool window and in the *Dimension Selector* tool windows.

2. Click the eye icon next to the *Probability map* layer in the *Layers* tool window once.
   - The probability map image now disappears from the image window. You see only the input image.
3. In the *Layers* tool window, click once on the *Probability map* image layer to select this image layer.
   - When you select an image layer in the *Layers* tool window, this image layer is automatically displayed.
   - The probability map is now displayed in the *Dimension Selector* tool window.

     The probability map is composed of several separate probability maps. The neural network computes an individual probability map for each training label class that has been defined as well as for the background. These different probability maps for the individual classes are displayed in the *Dimension Selector* tool window. The *Background* probability map is automatically hidden.

     In this example the deep learning analysis produces a probability map that is composed of two other probability maps, the *Background* and the *Cell nuclei* probability maps. The name and the color of the probability map are adopted from the training label class.



In the illustration, the image resulting from the neural network analysis can be seen on the left. In the resulting image, the input image and the probability map are superimposed on each other. On the right, in the *Layers* tool window (**1**) the probability map is selected. In the *Dimension Selector* tool window you can see that the probability map contains 2 other probability maps: *Cell nuclei* (**2**) and *Background* (**3**). The background is hidden by default.

4. Probability maps are monochrome images that are colored with the same color as the training label class.

   You can change the color of the probability map in the *Dimension Selector* tool window. In the *Dimension Selector* tool window, click on the color field to the right of the *Cell nuclei* channel. You can select the color blue from the palette to color the cell nuclei in the probability map blue, for example.
5. The brighter the intensity of a pixel in the probability map is, the higher is the probability that the pixel belongs to the relevant class.

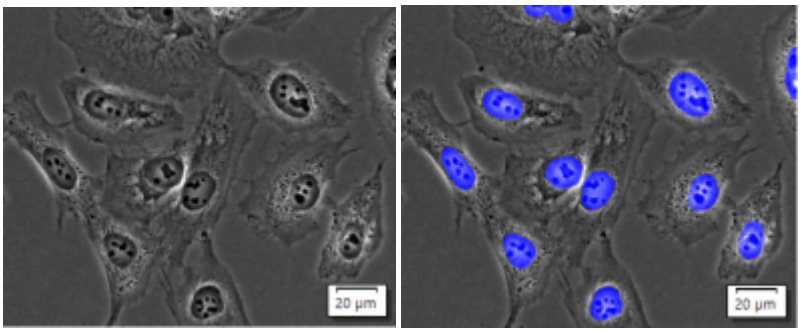
   In this example, almost no differences in intensity can be seen in the probability map within the cell nuclei. This means that the neural network has identified the cell nuclei with a very high degree of probability.

Saving resulting images

1. Use the *File > Save As* command to save the image that results from a Deep learning object analysis.

Step 5: Carrying out an object analysis

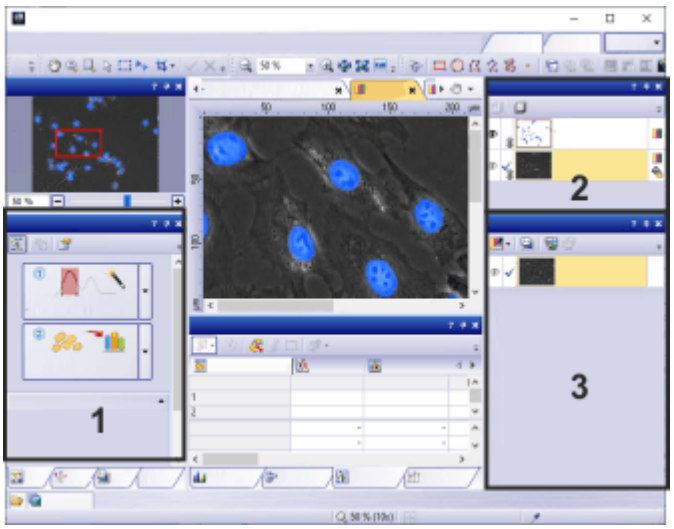
Example: Automatically determine the number of cell nuclei on a phase contrast image. Use the images that you have analyzed in step 4 using the neural network *NN_Nuclei_PH*.



The left image shows the phase contrast image on which the cell nuclei are to be counted. The right image shows the image resulting from an analysis using the *NN_Nuclei_PH* neural network. The probability map is superimposed on the phase contrast image. The cell nuclei were recognized correctly and are colored by the probability map. In this case, the probability map uses the color blue. To count the objects you need to perform an automatic object analysis on the probability map.

Preparations

1. Load the phase contrast images that you have analyzed using the *NN_Nuclei_PH* neural network.
2. Switch to the *Count and Measure* layout. To do this, use, for example, the *View > Layout > Count and Measure* command.
   - In the *Count and Measure* layout, the tool windows needed for an object analysis using thresholds are displayed by default.



In the *Count and Measure* layout, the tool windows *Count and Measure* (**1**), *Layers* (**2**) and *Dimension Selector* (**3**) are displayed by default.

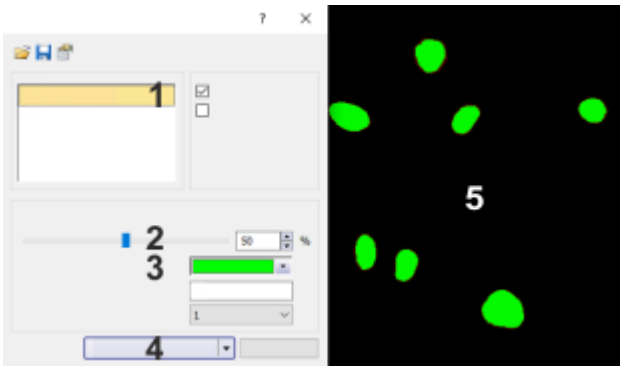
Carrying out an object analysis

1. In order for the automatic object analysis using thresholds to be successful, all of the objects must have the same color or intensity, and be clearly distinguished from the background. This is exactly the case for the probability map. The color of the cell nuclei is clearly different from the background.

   Select the probability map in the *Layers* tool window to perform the object analysis on the probability map.

2. In the *Count and Measure* tool window, click the small arrow next to the threshold setting button. The button displays the number 1. In the menu, select the *Probability Layer Segmentation* command to open the *Probability Layer Segmentation* dialog box.
   - This *Probability Layer Segmentation* command is only available when the probability map in the active image has been selected. If the active image doesn't have a probability map or if the probability map is not selected, the command is grayed out.
   - In the *Probability Layer Segmentation* dialog box, the channels of the probability map are listed in the *Channel* group. The *Background* channel is not listed by default.

     In this example, in the *Channel* (**1**) group only the *Cell nuclei* channel is listed.
   - In the image window (**5**), the cell nuclei are displayed in the color that has been selected in the *Color* (**3**) field.



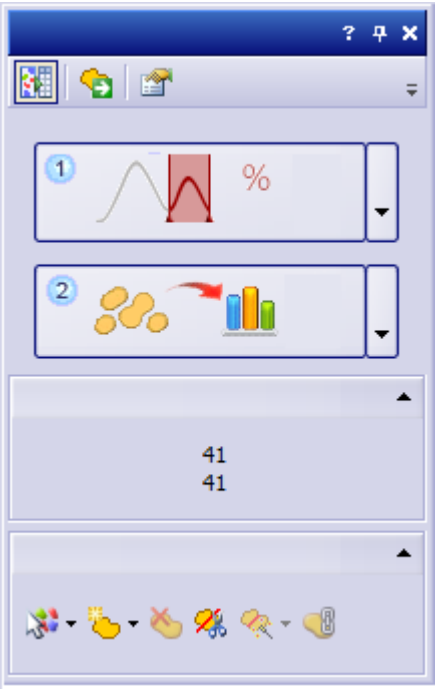
On the left, the illustration shows the *Probability Layer Segmentation* dialog box, and the image window with the detected cell nuclei on the right.

3. With object analyses on probability maps, a detection threshold is defined for each channel. All of the pixels that have a higher probability than this threshold will belong to an object. All of the pixels that have a lower probability belong to the background.

   Move the *Detection threshold* (**2**) slide control to see how changing the detection threshold affects the image.
   - When you increase the detection threshold, the detected objects become smaller. This enables you to exclude objects that do not have a high probability of being a cell nucleus.

- The detection threshold can help to separate neighboring objects that are very close to each other and have been mis-identified as a single object.

4. To carry out the object analysis, click the *Count and Measure* button (**4**) in the *Probability Layer Segmentation* dialog box.
   - The *Probability Layer Segmentation* dialog box is closed.
   - The automatic object analysis is only carried out on the *Cell nuclei* channel. The *Background* channel is hidden by default. This channel is not considered for the object analysis.
   - The objects found by the automatic object analysis are on the *Detected Objects* image layer. You can show and hide this image layer in the *Layers* tool window.
   - The number of objects found is displayed in the *Object Count* group in the *Count and Measure* tool window.
   - All of the objects will be automatically measured. Use the *Count and Measure Results* tool window to view the measurement results.
5. You can still edit the detected objects now. You can delete objects and exclude them from the classification for example. To do this, use the buttons in the *Edit Objects* group in the *Count and Measure* tool window.



The number of objects detected will be shown below, in the *Count and Measure* tool window, in the *Object Count* group. Should the *Object Count* group not be visible, click the small black arrow in the group's header to display it.

12.2.3. Additional Step-by-step instructions

Creating training labels manually

All of the images that you want to use to train a neural network must have at least two image layers. One image layer contains the image that was acquired of the objects you want to identify. The other image layer contains the training labels that clearly define

for the software the objects that you want to identify. You can manually draw the training labels on the image.

Example: Lets say you want to train a neural network that detects cell nuclei and cell tissue. Draw the required training labels on the images.

1. Load the first image on which you want to define the training labels.
2. If the *Training Labels* tool window isn't displayed, use the *View > Tool Windows > Training Labels* command to show it.
3.  In the *Trainings Labels* tool window, click the *New Training Label Class* button.
   - The last used training label classes will be overwritten.
   - A new training label class is created. The training label class is called *Class1* and the color of the first training label class is red.
   - When you create a training label class, the *User Labels* image layer is added to the active image. All training labels will be defined on this image layer.
4. Enter a name for the training label class. To do this, double click the *Name* name cell in the table in the *Training Label Classes* group. For this example, name the training label class *Tissue*.
5. If necessary, select the *Tissue* training label class in the *Training label Classes* group. Click one of the buttons in the *Training Labels* group to switch to the corresponding edit mode.

    For example, click the *Create Training Labels - Fill* button to automatically fill the training label that you draw.
   - The button becomes active, indicating which edit mode is active.
   - The edit mode remains active until you explicitly end it.
6. While pressing the left mouse button, outline all of the tissue in the image. You don't have to be completely exact when you do this. For a lot of tasks, it's ok to just outline the object roughly. This applies particularly when you are drawing background objects.
   - In this drawing mode, the training labels are automatically filled as soon as the ends of the line meet.
   - The training label is drawn in the *User Labels* image layer.
   - The color of the training label corresponds to the color of the class to which it belongs.
   - The training labels are transparent when they are drawn. This ensures that the objects under the training label remain visible.
7. Check the training labels you have drawn. It is important for the quality of the neural network that the training labels have been correctly defined. Depending on the task, it can be useful to have another expert check the training labels.

   You can at any time correct the training labels that have been drawn. You can delete whole training labels or just parts of them. You can expand training labels and you can draw new training labels. To do this, select the training label class and use the buttons in the *Training Labels* group.
8. Now define the second training label class, *Cell nuclei*.

To do this, in the *Trainings Labels* tool window click the *New Training Label Class* button again.

You can change the default color for the training label class. Click on the color field and select, for example, the color yellow for the training label class.

9. Draw the training labels for the cell nuclei on the image.

   If the training labels that have already been drawn are in the way, click the eye icon in the row for the *Tissue* training label class. This will hide the training labels that define the tissue. Click in the *Visible* cell to show the training labels again.

10. If you want the cell nuclei to belong to the tissue in later measurements, select the *multi-label classification* option in the *Training Label Layer* group. If you don't want the cell nuclei to belong to the tissue, select the *multiclass classification* option.

    Select one option and then the other and observe the effect that the selected classification has in the image window.

    - When you select the *multi-label classification* option, training labels can overlap. A pixel can then belong to more than one training label class simultaneously.

      All of the training label classes are displayed in the image window. When training labels overlap, you can see the order of the training labels in the image window. The training label class that has been created last will be located on top. In the list, it is located at the bottom.

    - When you select the *multiclass classification* option training labels can still overlap, but in the resulting image a pixel can now belong to one and only one training label class.

      Now you can no longer see all of the training labels in the image window. When a training label is covered by a different training label it is no longer visible. This setting affects the way the neural network is trained. If you want the neural network to detect the cell nuclei, the *Cell nuclei* training label class must be at the bottom of the training label classes list. Use the arrow buttons to change the order of the training label classes and observe the effect in the image window.

11. In this example you could also define a training label class for the background. To do this, just roughly outline some areas in the image background. Select the *Background* check box for this training label class.

12. Save the training label classes that have been defined. To do this, click this button in the *Training Label Classes* group. Save the parameter set with the name *Cells*.

    When you train a neural network, the same training label classes must be defined in all of the training images. When you have saved a parameter set, it makes it faster to label additional training images.

13. Save the training image with the training labels that have been defined. To do so, use the *File > Save As* command.

14. Load the next training image.

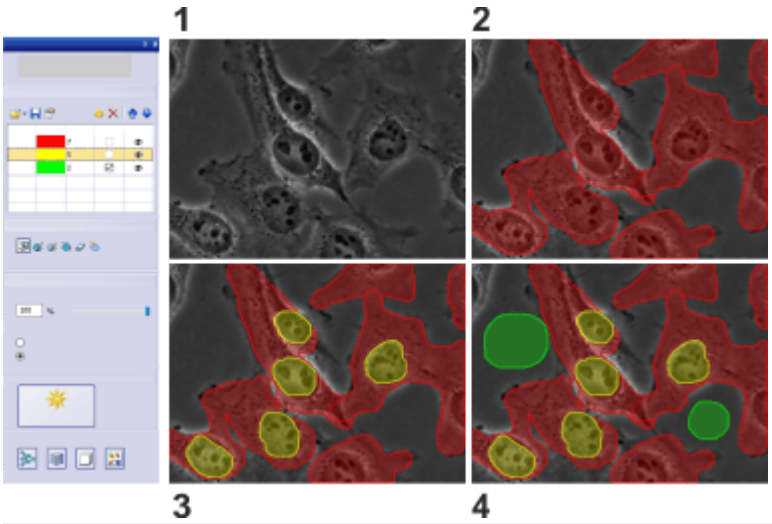
15. Click this button in the *Training Label Classes* group and load the *Cells* parameter set.

- The three training label classes that have been defined, *Tissue*, *Cell nuclei* and *Background* are loaded.

15. Select one of the training label classes and draw the appropriate training labels in the image.

    When you train a neural network, the same training label classes must be defined in all of the training images. You don't have to define a training label for every training label class. The number of training labels in this training label class will then be 0. This enables you to label training images that show growth, for example. Some training images have only young cells and others have only old cells.

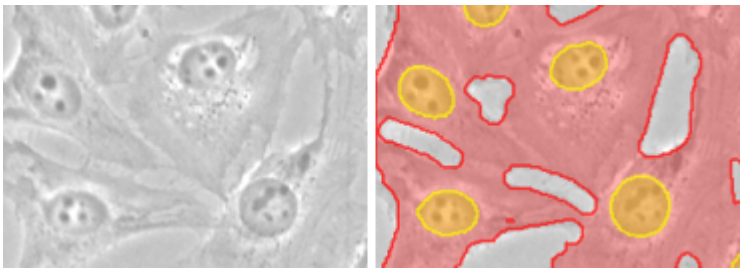
16. Release the *Create Training Labels - Fill* button in the *Training Labels* tool window to leave edit mode.
17. Save the training images.
    - You can now use the training images to train a neural network.



On the left you can see the *Training Labels* tool window with three training label classes. Image (**1**) shows the training image without any training labels. In image (**2**) the training labels for the tissue are shown. In image (**3**) the cell nuclei are labeled as well. The cell nuclei are on top of the tissue. The option *multiclass classification* detects cell nuclei and tissue separately. In image (**4**) the back has been roughly indicated.

Analyzing images that have more than one training label class

Example: Lets say you want to analyze samples that have two classes of objects. In this example, we want to measure the area fraction of the cell nuclei in relation to the rest of the cell tissue.
Use the *Deep Learning* software solution to train a neural network that detects two object classes in the image; *cell nuclei* and *cell tissue*. Then measure the area occupancy for the two object classes.



On the **left** of the illustration is one of the images that you want to analyze. **On the right** the training labels have been defined. There are two training label classes *cell nuclei* (yellow) and *cell tissue* (red).

Step 1: Creating training labels

1. Draw the training labels on the training images. You can use the *Training Labels* tool window to do this. Define two training label classes, *cell nuclei* and *cell tissue*.
2. In the *Training Labels* tool window, check the order of the training label classes. The *cell nuclei* training label class must be below the *cell tissue* training label class in the *Training Label Classes* group. In the image window, the cell nuclei will now be on top of the training labels for the cell tissue.

   If necessary, use the arrow buttons in the *Training Labels* tool window to change the order of the training label classes.
3. Save the training images.
   - You can now use the training images to train a neural network.

Step 2: Training and saving a neural network

1. Switch to the *Deep Learning* layout.
2. Click the *New Training* button.
3. Make the necessary settings in the *New Training: Input and Output* dialog box.

   Open the training images.

   Select the *Input channels > Basic mode* option.

   Make sure that the check boxes next to the *cell nuclei* and *cell tissue* classes are marked in the *Training Label Classes* group. This way both classes will be taken into account by the training process.

   In this example a pixel can't belong to the cell nucleus and to the cell tissue simultaneously. For this reason, select the *multiclass classification* option in the *Training label classes* group. The neural network will now allocate each pixel to the class for which its probability is the highest.
4. Click the *Next* button to select the model for the neural network.

For this example, select the *Standard Network* parameter set.

5. Click the *Start* button to start the training process.
6. Wait until the neural network training is finished.
7. Select the checkpoint at which the similarity is the highest. As a rule, it will be the last checkpoint.

   Save the neural network. To do this, click the *Save Neural Network* button. Give the neural network the name *2Classes*.

Step 3: Performing an analysis using the neural network

1. Load or acquire the images you want to analyze.
2. Open the *Options* dialog box by clicking the *Count and Measure Options* button, located in the *Count and Measure* tool window.
3. Click the *Count and Measure > Classification* entry in the tree view. Select the *Phase* classification scheme. Now all of the cell nuclei that are detected by the neural network will be allocated to the *cell nuclei* object class. The cell tissue that is detected will be allocated to the *cell tissue* object class.
4. Click the *Count and Measure > Detection* entry in the tree view. Select the *Borders - frame > Truncate* option. Now even objects that touch the edge of the image will be measured.

   Make sure that the *Fill holes* check box has been selected.
5. Select the measurement parameters for the phase analysis.

   In the tree view, click the *Count and Measure > Measurements* entry.

   Click the *Select Object Measurements* button to select suitable measurement parameters for the object measurement. Select the *Area* measurement parameter and close the dialog box.

   Click the *Select Class Measurements* button to select suitable measurement parameters for the class measurement. Select the *Object Class*, *Sum (Area)* and *Area Fraction Objects* measurement parameters. Close the dialog box.

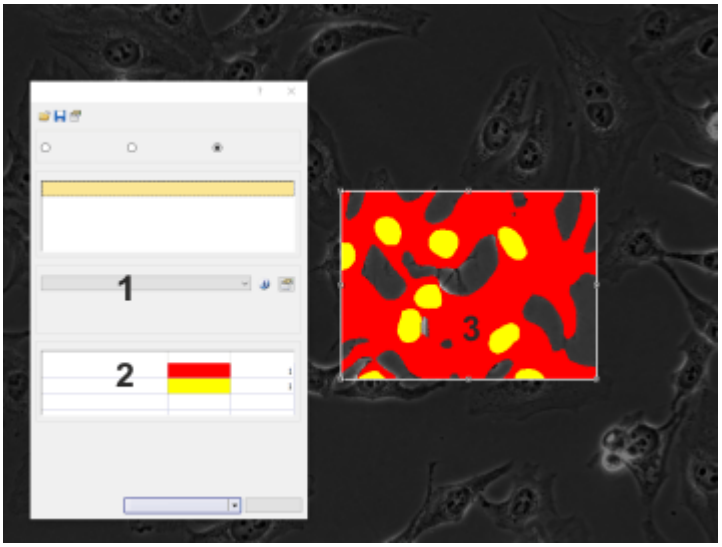
   Close the *Options* dialog box.
6. In the *Count and Measure* tool window, click the small arrow next to the threshold setting button. The button displays the number 1. Select the *Neural Network Segmentation* command in the menu to open the *Neural Network Segmentation* dialog box.
7. Select the *Not used* entry from the *Neural network* list.

   The preview area that is currently defined in the image window will be analyzed. You can accelerate the analysis significantly by reducing the size of the preview area. To do this, in the image window drag one of the preview area's handles inwards. This is quicker when no neural network is currently selected.
8. In the *Neural Network* list (**1**) select the *2Classes* neural network.

   In the list appear only neural networks that were trained on a single input channel and for which no Z-stack handling has been defined.

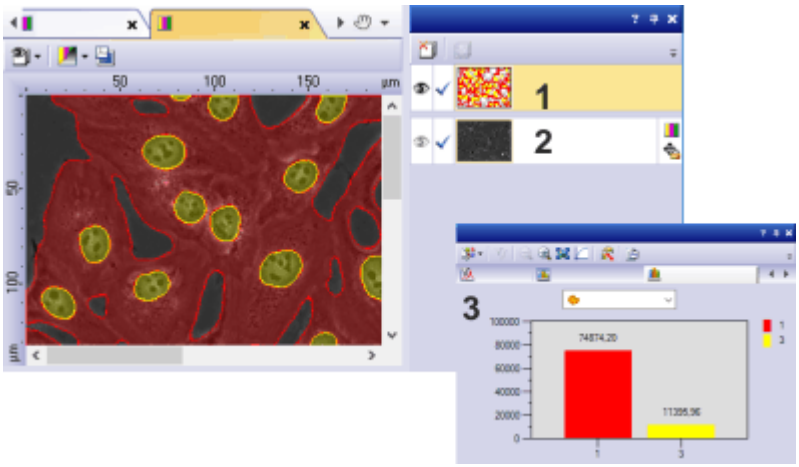
- In the *Neural Network Segmentation* dialog box, the training label classes are listed in the *Phases* (**2**) group. In this example there are two training label classes, *cell nuclei* and *cell tissue*.
- Your software starts the analysis as soon as a suitable neural network has been selected. The analysis can take several minutes. Pay attention to the progress bar located in the status bar.
- In the preview area (**3**) you can see which parts of the image have been allocated to the cell tissue and which parts have been allocated to a cell nucleus. For the display, the colors that are currently selected in the *Color* field are used.

Note: You can accelerate the computation of the preview image by reducing the size of the preview area in the image window. To do this, drag one of the preview area's handles towards the center.



The illustration shows the *Neural Network Segmentation* dialog box and the preview window.

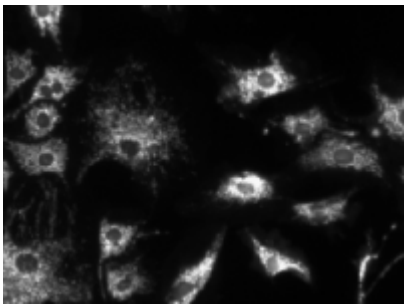
8. Click the *Count and Measure* button to get the results.
   - The results are displayed in the *Count and Measure Results* tool window in the *Class Measurements* results view. You can see the area that cell nuclei and cell tissue take up in the image.



The results of the object analysis using a neural network: The image that was analyzed (**2**) now has the *Detected Objects* image layer (**1**) which contains the objects that were detected. The results table shows the area of the cell nuclei and the area of the cell tissue. The class histogram (**3**) shows the area distribution in a bar chart.

Analyzing images using live AI

Example: Let's say you want to count cell nuclei in the live-image.



You want to count the cell nuclei at different positions on the sample in an image like this one.

1. Create or import a suitable neural network. The training images for the neural network should have been acquired with as similar acquisition conditions as possible.

   To achieve a high frame rate in *Live AI* mode, train the neural network using the *Fast Network* training configuration.

   In this example we want the neural network to detect cell nuclei in a fluorescence image.
2. Make the *Camera Control* and *Live AI* tool windows appear. To do this, use the appropriate commands in the *View > Tool Windows* menu.

   Show the *Camera Control* toolbar. To do this, use the *View > Toolbars > Camera Control* command.
3. Use the *Tools > Options* command. Select the *Count and Measure > Display* entry in the tree view. Select the *Object display > Outline* option.
   - Now only the outlines of the detected cell nuclei are shown in the image window so that they cover as little image information as possible.
4. Before starting *Live AI* mode, optimize the image quality. In the *Camera Control* tool window, click the *Live* button to switch to live mode.

You can use the *Toggle subarray* function. This crops the image that your camera acquires and the neural network analysis is quicker.

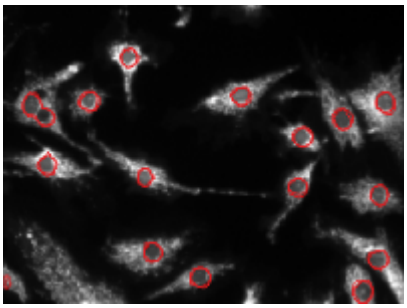
Then, in the *Camera Control* tool window, select the optimal settings for the image acquisition.

Finish the live mode. To do this, click the *Live* button again in the *Camera Control* tool window.

5. In the *Live AI* tool window, select the neural network that you want to use. There is a list of available neural networks in the *Neural Network* group.

   Select the *Object counting* option in the *Settings* group.

6. In the *Live AI* tool window, click the *Live AI*  button.
   - *Live AI* mode starts.
   - The *Live AI*  button in the *Live AI* tool window changes its appearance, showing that *Live AI* mode is active.
   - The live-image is displayed in the image window.
   - Most of the other functions in your software are no longer available when you are in *Live AI* mode. For example, you can't change any settings in the *Camera Control* tool window when you are in *Live AI* mode.
7. Wait until the neural network analysis is finished.
   - The detected cell nuclei are now displayed in the image window.
   - In the *Live AI* tool window, the *Results* group displays the number of cell nuclei that are currently visible in the image.



After the neural network analysis, the cell nuclei in the image are detected and counted.

8. Go to a different position on the sample and wait until the neural network analysis of this position is finished.
9. If you want to change the exposure time in the live-image, use the *View > Toolbars > Camera Control* command to show the *Camera Control* toolbar.

   Enter the required exposure time in the input field on the toolbar or use the arrow buttons.
10. If you want to save an image together with the result of the analysis, click the *Snapshot*  button in the *Live AI* tool window.

- A new image document is created in the document group. The image document contains the acquired image together with the *Detected Objects* image layer.

11. Switch off *Live AI* mode. To do this, click the *Live AI* button again.

- The *Live AI*  button in the *Live AI* tool window now has a green arrow again.
- In the *Count and Measure* tool window you can now view the number of cell nuclei that have been counted for this image

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12.3. Tracking objects

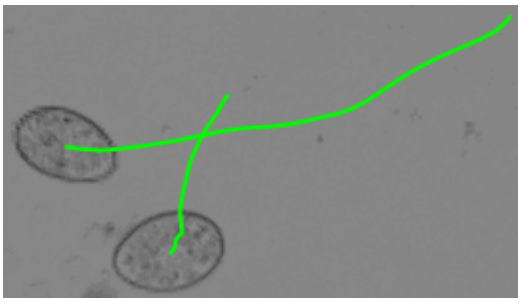
12.3.1. Overview - Tracking objects

You can track and analyze the movement of objects with your software (cell movement for example). Many objects can be tracked at the same time. The tracked objects can branch, or two objects can merge into one.

How are the physical objects tracked?

Tracking objects automatically

Acquire a time stack or a movie of physical objects that are moving. The movement of the objects is usually tracked automatically. First, all of the objects in the frames of a time stack are detected. Your software decides which of the detected objects belong together and connects them in a track. An object's track shows the path that a physical object took during the recording time.



The illustration shows the first image in a time stack. You can see two cells and their tracks.

Tracking objects manually

If you only want to track a few objects, you can track their movements manually. With manual object tracking, you specify the position of the object yourself using the mouse.

Performing object tracking

The *Object Tracking* tool window contains all of the functions that you need to track objects. Perform the steps in the *Object Tracking* tool window in the order that they appear.

What do you get when you track objects?

When object tracking has been performed successfully, certain results are displayed in the image window. You can see the tracks of the physical objects as well as all of the objects that were detected in the frame that is currently displayed in the image window. There are several ways of coloring the tracks and objects.

All detected objects and tracks can be measured and classified. You can view the tables with the precise measurement results as well as charts that assist in visualizing the measurement results in the *Object Tracking Results* tool window.

The results of object tracking are saved together with the image. This means that you don't have to save the results sheets separately. After performing object tracking, save the time stack as a TIF or a VSI file. The results will then be loaded in your software the next time you load the time stack.

Exporting results

The data in the *Object Tracking Results* tool window can be exported to a workbook or to a chart. You can also export the results sheets to MS-Excel to process the data further.

Prerequisites for object tracking

What software do you need?

The object tracking functions are only available when the *Object Tracking* solution has been purchased.

You also require the automatic object analysis functions. These functions are available when you purchase the *Count & Measure Full* software solution. Please note that your software has two different software packages for automatic object analysis. You require the more advanced software package *Count & Measure Full*.

What type of images can objects be tracked on?

Object tracking requires a time stack in which the movement of objects can be clearly recognized.

You can create time stacks using the *Movie* or *Time Lapse* acquisition processes. The frames making up a time stack can be 8-bit gray-value images, 16-bit gray-value images or 24-bit true-color images. AVI videos can also be used as a source for object tracking.

You can also track objects on a multi-channel time stack. In this case, objects that belong together are detected on all color channels and a track is allocated to them. For example, you can track objects that change their fluorescence color during the image acquisition.

Prerequisites for the source image

Not all time stacks are suitable for object tracking. Please note the following requirements:

- The objects that you want to track must not be connected, but must be clearly separated from one another. The objects are in the foreground of the image and

should be visually distinct from the background of the image.
- The sample must be in focus when the time stack is acquired.
- The sample should be as evenly illuminated as possible. If necessary, check the shading correction.
- The interval of a time stack is the time interval between two consecutive frames. The interval should match the speed of the movement of the objects so that each frame displays a clear development in the movement of the physical object, however this step should not be too large.
  The intervals in the time stack have to be equal to enable object tracking. This means that all of the frames have the same time interval between them.
- There is no limit to the number of objects. However, a large number of objects leads to very many measurement results and increases the time required for object tracking.
- The images must be correctly XY-calibrated. Only then can the tracks be correctly measured. If necessary, use the *Manual Magnification Calibration* calibration process.
- The stage should not move while the time stack is being acquired, otherwise the movement of the objects will be distorted.

The procedure for object tracking

The procedure for automatic object tracking

The steps printed in bold have to be performed each time object tracking is performed. The other steps are optional. You will usually adopt the settings that you used for the last object tracking that was performed.

Preparing for object tracking

Load a time stack that shows the objects that you want to track. Take a look at the time stack to get an overview of the type and number of objects to be tracked.

If necessary, process the time stack to enable the objects to be detected more easily.

Performing object tracking

You can use the *Object Tracking* tool window to track moving objects. Perform the steps in the *Object Tracking* tool window in the order that they appear. Select a suitable scenario, detect and filter objects on the frames, and compute tracks.

Defining, viewing and saving the results

Select the required measurement parameters for the tracks and objects that have been detected. Use the *Object Tracking Results* tool window to view the results.

You can export the results as a table or a chart.

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12.3.2. Tracking fast objects

Task: Acquire a time stack of moving cells. You want to know how quickly the cells are moving and how far they travel during the course of the time stack.

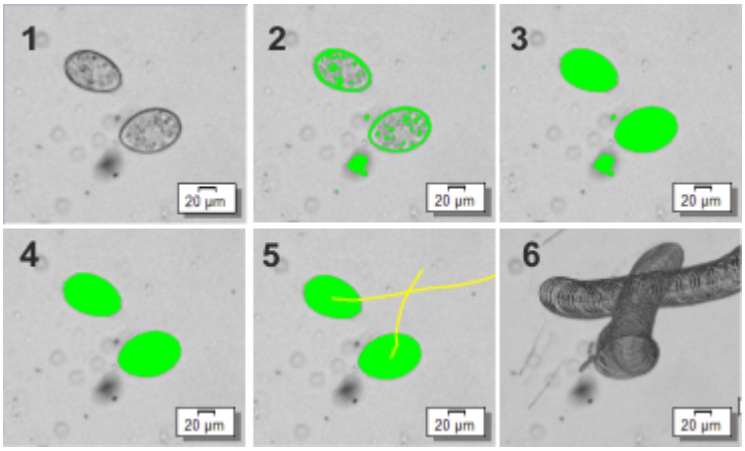


Image (**1**) shows a frame in the time stack that contains two cells. First, threshold values are defined to detect cells (**2**). You can fill holes in the detected objects (**3**). The detection process also finds objects that aren't cells. An object filter (**4**) excludes all of the objects that aren't cells. The tracks (**5**) show the course of the cells' movement. Image (**6**) shows the projection image for the whole time stack. You can use this image to check the accuracy of the object tracking process. The minimum intensity projection also shows the cells' tracks.

The following process flow chart displays the basic steps of the process.

Preparing for object tracking

Performing object tracking

Perform the steps in the *Object Tracking* tool window in the order that they appear.

Defining, viewing and saving the results

Select the required measurement parameters for the tracks and objects that have been detected. Use the *Object Tracking Results* tool window to view the results.

You can export the results as a table or a chart.

Preparing for object tracking

1. Load or acquire a time stack that shows the moving objects that you want to track.
2. Take a look at the time stack to get an overview of the type and number of objects to be tracked. To do this, use the navigation bar in the image window. You can play back the time stack like a movie or you can move manually from one frame to the next.
3. Use the *View > Scale bar* command to display a scale bar in the image window.

Performing object tracking

4. Use the *View > Tool Windows > Object Tracking* command to open the *Object Tracking* tool window.

The *Object Tracking* tool window contains all of the functions that you need to track objects. Some of the steps are optional. In this scenario, the following steps are required:
(**1**) *Movement Scenario*, (**2**) *Detect Objects*, (**3**) *Filter Objects*, (**4**) *Define Track Linking*, (**5**) *Start Tracking*

5. The cells move roughly on a straight course. They don't split or merge. Select the *Fast objects* entry in the *Movement Scenario* list.

Detecting objects

6. In the *Object Tracking* tool window, click the *Detect Objects* button to detect and measure objects on all of the frames in the current time stack.
   - All of the objects that have been detected will be displayed in color.
7. The object analysis is performed using the current settings. Check whether the objects have been correctly detected.

   If the objects have not been correctly detected, define thresholds for the current image and check the detection settings.

Filtering objects

8. Tracking a lot of objects requires a lot of calculation time. For this reason, exclude as many objects as possible that are not relevant. This reduces the calculation time and makes it easier to get a clear overview of the results.

Defining track linking

9. Define some of the settings for allocating objects to a track. To do this, click the arrow to the right of the *Start Tracking* button to open a context menu. Select the *Define Track Linking* command from the context menu.
10. Select the search range. The search range has a strong effect on the results of the object tracking process. Your software only searches within the search range for

the next object in a track. If the object tracking process is not successful, try changing the search range. Start with as small a search range as possible.

You can try selecting the *Search range > Absolute* option. Enter the required value in the field. The length of the scale bar in the image gives you an indication of an appropriate value.

11. Close the *Define Track Linking* dialog box.

Starting the object tracking

12. Start object tracking. To do so, click the *Start Tracking* button in the *Object Tracking* tool window.
    - The movement of the objects is tracked automatically.
    - Depending on the number of objects, it may take some time before all of the tracks are detected and measured. Pay attention to the progress bar located in the status bar. You can abort the process at any time by clicking the *Cancel* button.
    - While your software is tracking the objects, you can use the other software functions. You can acquire the next time stack for example.
    - When the object tracking is complete, the tracks are shown in the image window.
    - The tracks are on their own layer in the image. This layer is called *Tracks*.

Defining, viewing and saving the results

1. Specify the color of the tracks in the image window.

   To do this, use the *Tools > Options* command. Select the *Tracking > Display* entry in the tree view.
   Select the *Fixed track color* option to display all of the tracks in the same color.

2. Use the *Set layer visibility* button to show or hide the tracks and the objects in the image window. You can find this button in the image window's navigation bar.
   - You can also use the *Layers* tool window to show or hide image layers. If you delete the *Tracks* layer, you automatically delete all of the object tracking results.

3. If the *Object Tracking Results* tool window is not displayed: Click the *Object Tracking Results* button in the *Object Tracking* tool window to display the *Object Tracking Results* tool window.

4. In the *Object Tracking Results* tool window, activate the *Track Measurements* results view.
5. Select the *Mean (Track Velocity)* and *Track Length* measurement parameters to measure the tracks.

6. Take a look at the results for the individual tracks in the *Track Measurements* results view in the *Object Tracking Results* tool window.

- The mean velocity of the objects and the track length are displayed in the table. This tells you how quickly the cells are moving and how far they travel during the course of the time stack.

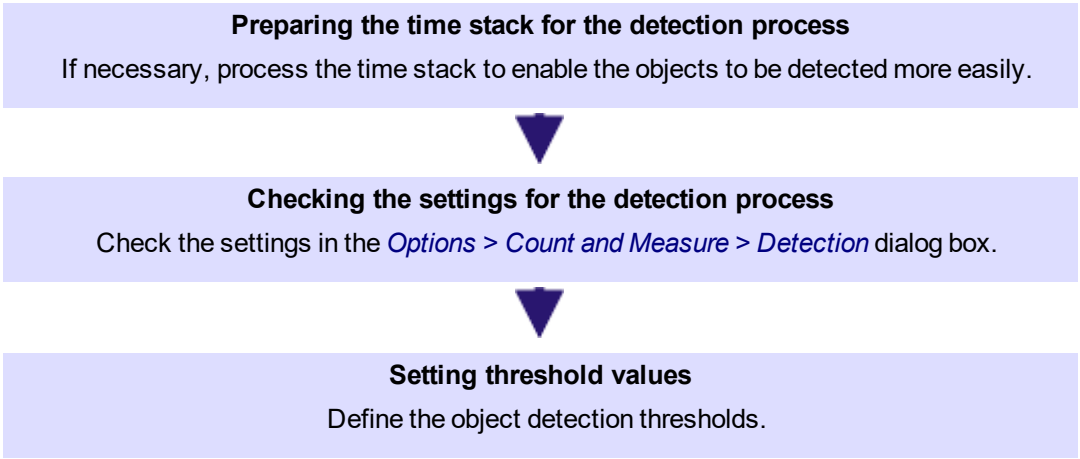
Saving the results of object tracking

7. Activate the image window. Select the *File > Save As* command and save the image together with measurement results. Select the *Tagged Image File Format (TIFF) (*.tif)* or the *Virtual Slide Image (*.vsi)* file format.

12.3.3. Detecting objects

In order for automatic tracking of physical objects to work successfully, the objects first have to be detected in the image. You can't track an object that hasn't first been detected. The process detects all of the objects in the frames of a time stack.

The following process flow chart displays the basic steps of the process.



Preparing the time stack for the detection process

1. The objects must be easy to detect. If required, edit the time stack that contains the objects using a command from the *Process* menu. You can perform background correction or shading correction or smooth the image, for example.

Checking the settings for the detection process

2. In the *Object Tracking* tool window, click the small arrow next to the *Detect Objects* button. Select the *Detection Options* command from the context menu to open the *Options > Count and Measure > Detection* dialog box.
3. Use a size filter to prevent small objects from being detected. To do this, you can enter a value of 10 in the *Minimum object size* field in the *Options* group, for example. An object must now be at least 10 pixels large in order to be counted as an object.
4. You can automatically fill the holes in an object. Select the *Fill holes* check box, if holes are to be ignored, and in this way, evaluated as part of the objects.

- The way that the holes are treated affects the center of gravity of the object, among other things. With object tracking, the track connects the centers of gravity of objects that belong together.

5. Click *OK* to exit the *Options* dialog box.

Setting threshold values

When setting threshold values, you determine the range in which the object's intensity or color must lie. An object is formed of contiguous pixels whose intensity or color is within a particular range.

1. Go to the frame on which you can recognize the individual objects most clearly.
2. In the *Object Tracking* tool window, click the small arrow next to the *Detect Objects* button. Select the *Automatic Threshold* command from the context menu to open the *Automatic Threshold* dialog box.
   - The dialog box displays a histogram for the active frame.
   - The threshold values are set automatically in the *Automatic Threshold* dialog box.
   - All of the objects that have been detected will be displayed in color.
3. Check whether the objects have been correctly detected.
   Should the objects not have been correctly recognized, go to the *Background* group and enter whether the background is bright or dark.
4. Click the *Count and Measure* button.
   - The *Automatic Threshold* dialog box will be closed.
   - Now all of the objects whose intensity is within the thresholds that were defined are searched for in all of the frames in the time stack. With many frames and many small objects, the detection process can take some time.
   - All of the objects that have been detected will be displayed in color. The color of the objects is determined by the current object classification. Use the *Options > Count and Measure > Classification* dialog box to select or to define a classification scheme.
   - The objects that have been detected are then displayed on their own image layer. This image layer is called *Detected Objects*. Use the *Layers* tool window to make these image layers appear or disappear, or to delete them.
5. As soon as the objects have been detected, you can start tracking the physical objects. To do so, click the *Start Tracking* button in the *Object Tracking* tool window.

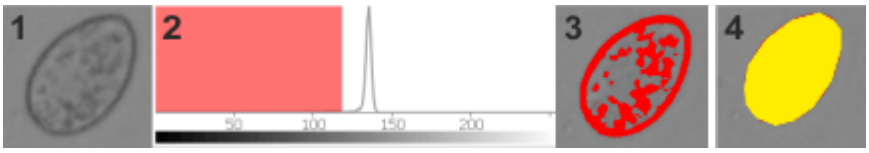


Image 1: The original image contains a cell.
Image 2: The histogram of the image shows the intensity distribution. Bright pixels have a high intensity and dark pixels have a low intensity. The red area corresponds to the threshold value range. All of the intensity values within the threshold values are defined as the foreground of the image.
Image 3: The automatic thresholds are set so as to put the objects in the foreground. As long as the threshold value dialog box is open, the objects in the image are displayed in the color that has been selected for the phase in the dialog box.
Image 4: When the detection process is finished, the objects in the image are displayed in color. The color of the objects now depends on the current object classification. In this example, the detection process has filled the holes.

12.3.4. Filtering objects

Before starting tracking, exclude as many objects as possible that are not relevant. This reduces the calculation time and makes it easier to get a clear overview of the results.

Task: Unwanted objects were detected in addition to the objects that you want to track in a time stack. Define an object filter that only includes objects of a certain size.

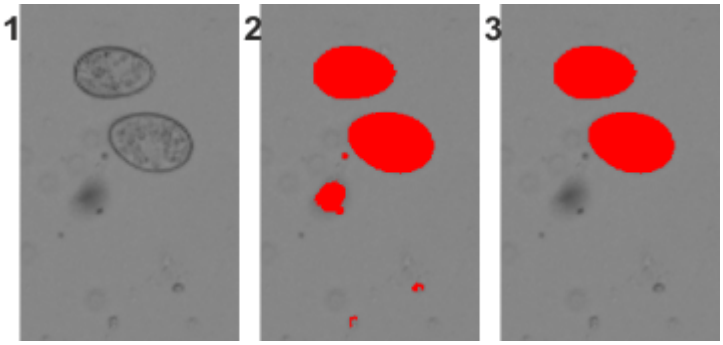


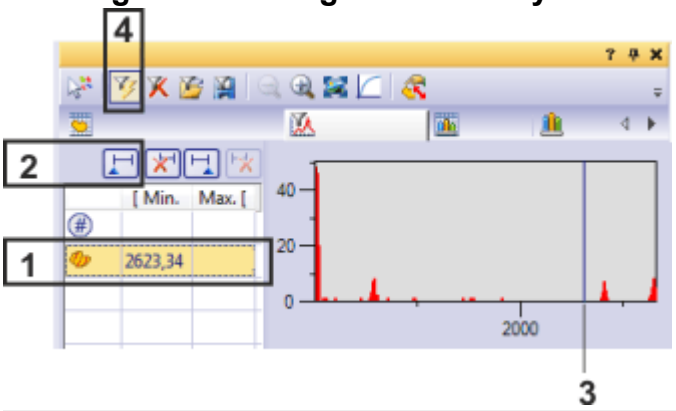
Image (**1**) shows two cells whose movements you want to track. In addition to the cells, the detection process found other objects that have the same intensity as the cells. The detected objects are colored red in the image (**2**). After an object filter is defined, only the two cells are detected in the image (**3**). The other objects have been excluded by the filter.

1. Load or acquire a time stack that shows the moving objects that you want to track.
2. Track the objects in the time stack.
3. In the *Object Tracking* tool window, click the *Filter Objects* button.
   - The *Count and Measure Results* tool window appears. The *Object Filter* tab is automatically activated.
   - The table on the left-hand side of the *Count and Measure Results > Object Filter* tab displays a list with all of the measurement parameters that are currently selected for the object analysis. The corresponding filter ranges are listed next to the measurement parameters.
   - If the measurement parameter that you want to use for the object filter doesn't appear in the list, click the *Select Object Measurements*

button. You can find the button on the *Count and Measure Results* tool window's toolbar.

If you want to filter by object area, for example, select the *Area* measurement parameter.

Defining the filter range interactively



Use the *Object Filter* tab in the *Count and Measure Results* tool window to exclude the objects that you don't want to track.

4. Select the *Area* (**1**) measurement parameter in the table.
   - A histogram with the area distribution of the detected objects is displayed to the right of the table containing the measurement parameters. This shows all of the objects, regardless of which frame they were detected on.

5. Click the *Select minimum value* (**2**) button above the list with the measurement parameters to define the minimum value for the filter range.
   - The mouse pointer changes shape.
6. In the image window, click on the smallest object that you want to include in the object tracking process.
   - The measurement value will then be automatically adopted in the *[Min.* field.
   - A line appears in the histogram (**3**). You can drag this line to the left and right in the histogram. This immediately changes the current minimum value.
   - In the image window, the result of the filtering of the objects can be seen straight away. All of the objects that are outside the defined filter range will be excluded.

- The *Toggle Object Filter* (**4**) button appears clicked, thereby showing you that the object filter is active.

Note: A defined object filter is not automatically deactivated when you load another image. If no objects are shown in an image, make sure that the object filter is deactivated.

12.3.5. Observing cell division

Task: Acquire a time stack of cells that are dividing. You want to know how the size of the cells changes before and after splitting.

1. Load or acquire a time stack that shows the moving objects that you want to track.
2. Take a look at the time stack to get an overview of the type and number of objects to be tracked.
3. In this example, the cells are not moving in any particular direction. They are staying more or less in the same position. Select the *Growing cells* entry in the *Movement Scenario* list.
   - The *Start Tracking* button now looks like this:

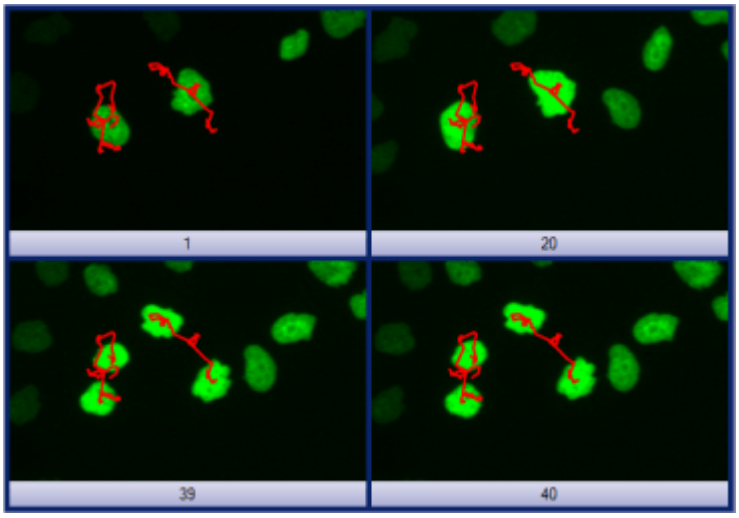
     
4. Detect the objects
5. Define some of the settings for allocating objects to a track. To do this, click the arrow to the right of the *Start Tracking* button to open a context menu. Select the *Define Track Linking* command from the context menu.

Defining track linking

6. Click the *Define search range* button to define the search range interactively in the image.
   - The mouse pointer will appear in the image window.
7. Click an object. Move the mouse to vary the size of the search range.
   - A circle is drawn in the image to help you to estimate a suitable size for the search range. Define a search range that also includes the cells after they have split.
8. Click a second time to specify the size of the search range.
9. Click the right mouse button and select the *Confirm Input* command in the context menu to confirm the search range that you defined.
10. Close the *Define Track Linking* dialog box with *OK*.

Starting the object tracking

11. Start object tracking. To do so, click the *Start Tracking* button in the *Object Tracking* tool window.
    - The movement of the objects is tracked automatically.
    - When the object tracking is complete, the tracks are shown in the image window.



When the object tracking process is complete, two tracks are shown in the image window. The illustration shows the found tracks and frames at different time points. As expected, the tracks branch once.

Defining, viewing and exporting the results

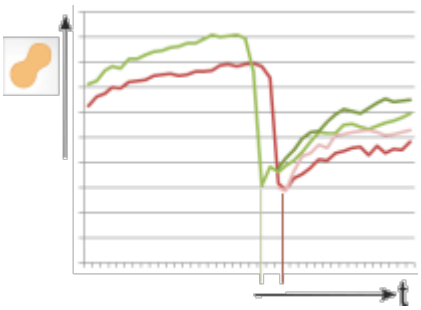
1. In the *Object Tracking* tool window, click the *Object Tracking Results* button to display the *Object Tracking Results* tool window.
2. In the *Object Tracking Results* tool window, activate the *Track Object Measurements* results view.
3. Select the measurement parameters *Track ID*, *Value (t)* and *Area*.

4. Take a look at the results for the individual tracks in the *Track Object Measurements* results view in the *Object Tracking Results* tool window.

   - The table now shows the area of each object and the time point of the frame in which the object was detected. This track ID tells you which track an object belongs to.

Exporting the results of object tracking

5. In the *Object Tracking Results* tool window, click the *Export to Excel* button to export the results into an MS-Excel file.
6. Give the sheet a descriptive name, then save it in the directory you want.
7. You can, for example, create a chart in MS-Excel that plots the area of the object against the time.

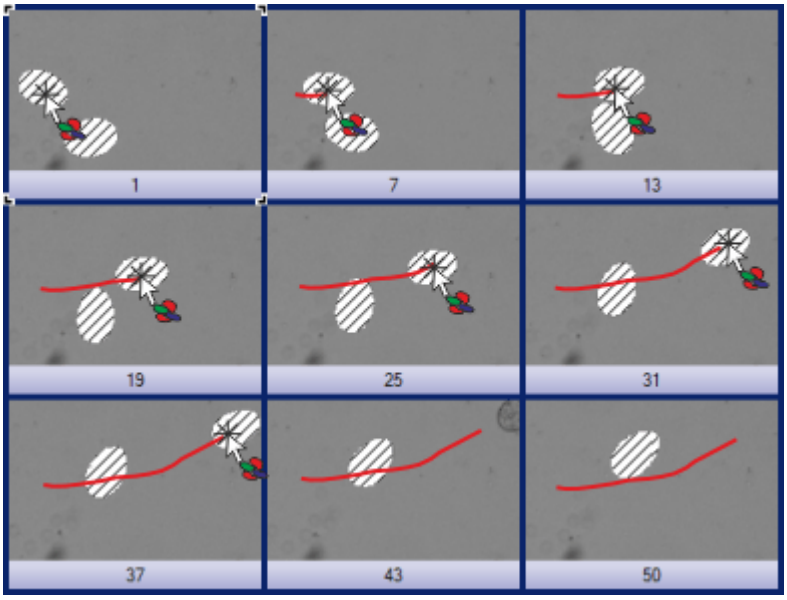


The chart shows how the size of the cells changes over time. The time point when the cells divide is clearly visible in the chart. The time points when the cells divide are slightly staggered.

12.3.6. Tracking objects manually

If automatic object tracking was unsuccessful, and you only have a few objects that you want to track, you can track them manually.

Task: Track a cell manually.

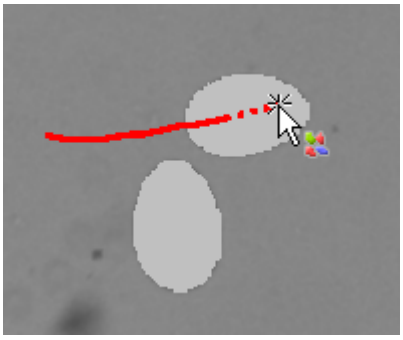


The illustration shows several frames of a time stack that has a total of 50 frames. The detected objects have a hatched pattern because they don't belong to any object class. The object has been clicked in each frame. You can see how the object's track gets longer from frame to frame. The track finishes in frame 37. In the last two frames the object has left the image area.

1. Load or acquire a time stack that shows the objects that you want to track.
2. Even when you are tracking objects manually, they first have to be detected in the frames. Perform an object analysis to detect the objects in the frames.
   - All of the objects that have been detected will be displayed in color.
3. In the time stack, go to the frame in which the object that you want to track first appears. To do this, use the navigation bar in the image window.
4. In the *Object Tracking* tool window, select the *Manual* entry from the *Movement Scenario* list.

- The *Start Tracking* button now looks like this:
  

5. In the *Object Tracking* tool window, click the *Start Tracking* button.
   - Your software will automatically switch to the selection mode. The shape of the mouse pointer on the image indicates the current mode.
6. Click the object that you want to track. You can only click an object that has already been detected. Where exactly in the object you click doesn't effect the object tracking process. A star appears at the tip of the mouse pointer when it is on an object.
   - The start point of the new track is automatically placed in the center of gravity of the selected object.
   - Your software automatically displays the next frame in the image window.
7. Click the object that you want to track in each frame.



The illustration shows a specific time point during the manual tracking of the upper object. The solid red line is the track of the object up until this point in time. The line from the current end of the track up to the mouse pointer is dashed. Because the mouse pointer is over an object, a star appears at the tip of the mouse pointer.

   - Your software displays the track of the object up to the current time point in the image window. The segment of the track that is being specified in the current frame is dashed.
8. Finish defining the track in the frame in which the object that you are tracking disappears. Click the right mouse button, and use the *Confirm Input* command in the context menu.
   - The results will be immediately updated. The newly added track is displayed in the image window and in the tables and charts in the *Object Tracking Results* tool window. The total number of found tracks is updated in the *Object Tracking* tool window.
   - A message box appears. You will be asked if you want to create more tracks.
9. Decide whether to finish the object tracking process or whether to track more objects. If you want to track more objects, click the *Yes* button in the message box.
10. In the time stack, go to the frame in which the new object that you want to track first appears. To do this, use the navigation bar in the image window.
11. If you want to track additional objects, note the following points:

- All existing tracks are automatically hidden in the image window when you track objects manually.
- As soon as you move the mouse pointer over an object that already belongs to a track, the track is shown.
- If you click an object that already belongs to a different track, you connect the two tracks. By doing this you automatically create a branch event.

12.3.7. Selecting measurement parameters

Task: Acquire a time stack of moving cells. You want to identify the 10 fastest objects. You also want to take a close look at the frame in which the objects are at their fastest. In addition to that, you want to export the results into a sheet.

Preparations

1. Acquire or load an image.
2. Track the objects in the time stack.

Selecting parameters for measuring tracks

3. In the *Object Tracking* tool window, click the *Object Tracking Results* button to display the *Object Tracking Results* tool window.

4. In the *Object Tracking Results* tool window, activate the *Track Measurements*  results view. Click the *Select Track Measurements* button.
   - The dialog box displays a list of all of the available parameters that you can measure on a track. At the bottom of the dialog box you'll see a list of the measurement parameters that are currently displayed in the *Track Measurements* results view in the *Object Tracking Results* tool window.
3. In the *Available track measurements* list, select the *Track Velocity* measurement parameter.

   - The *Track Velocity* measurement parameter tells you the velocity of the tracked object over the whole length of the track.

     The velocity of tracked objects is usually not constant. The velocity between consecutive frames is determined. This means that you can only compute one statistical value for the velocity of the track.
4. Click the *Mean* entry in the list under the drawing to select the *Mean (Track Velocity)* measurement parameter. This determines the mean velocity of the objects.
5. Click the *Add 'Mean (Track Velocity)'* button.
   - The measurement parameter appears in the *Measurements computed for all tracks* list. All of these measurement parameters will be displayed in the tool window.

6. Select the *Track ID* measurement parameter as well.

- The *Track ID* measurement parameter is a unique ID number for the track.

7. Close the *Select Track Measurements* dialog box with *OK*.
8. Take a look at the results for the individual tracks in the *Track Measurements* results view in the *Object Tracking Results* tool window.
   - The mean velocity of the objects is now displayed in the table. This tells you how fast the objects are moving.

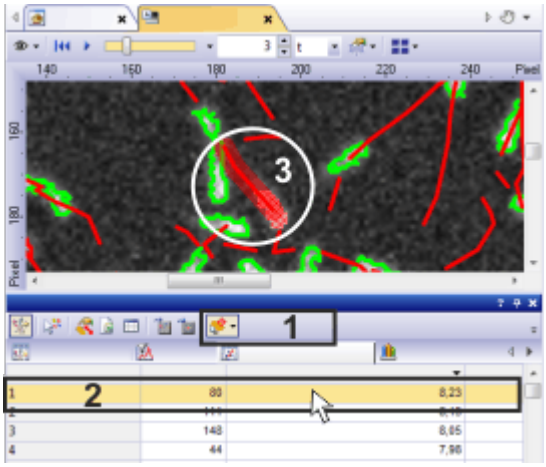
Selecting parameters for measuring objects

1. In the *Object Tracking Results* tool window, activate the *Track Object Measurements* results view. Click the *Select Track Object Measurements* button.
   - The dialog box displays a list of all of the available parameters that you can measure on the objects. At the bottom of the dialog box you'll see a list of the measurement parameters that are currently displayed in the *Track Object Measurements* results view in the *Object Tracking Results* tool window.
3. Select the measurement parameters *Track ID* and *Object Velocity*.

   - The *Object Velocity* measurement parameter tells you the current velocity of the object. The velocity between the previous and the subsequent frame is measured.
4. Close the *Select Track Measurements* dialog box with *OK*.
6. Take a look at the results for the individual objects in the *Track Object Measurements* results view in the *Object Tracking Results* tool window.
   - The velocity of the objects is now displayed in the table. This tells you how fast the tracked objects were moving at different points in time.
7. Sort the table by object velocity to find out which object is the fastest. To do this, double click the header of the column that contains the *Object Velocity* measurement parameter.
   - An arrow in the header will show you the direction in which they are sorted. The arrow should be pointing downwards .
   - The measurement values in the *Object Velocity* column are now sorted from high to low. The fasted detected object is now in row 1.
   - The *Track ID* measurement parameter in the other column tells you which track the object belongs to.

Finding the fastest object in the image window

1. Click one of these two buttons  to open a context menu. You can find the buttons in the tool window's toolbar. Select the *Pan and Zoom to Object* command to switch to a selection mode.
   - The button becomes active, thereby showing you that one of the modes is active.
   - The button displays the icon for the mode that is selected.
2. Select the first row in the *Track Object Measurements* results table to view the fastest object.
   - The fastest object will be selected in the image window.
   - If only a part of the image is displayed in the image window: The image segment displayed in the image window automatically moves so as to show the object. The zoom factor is changed to display the object and the track in their entirety.
   - The object's track is also selected.
   - If your object isn't in the current frame, your software automatically goes to a frame that does contain the object.

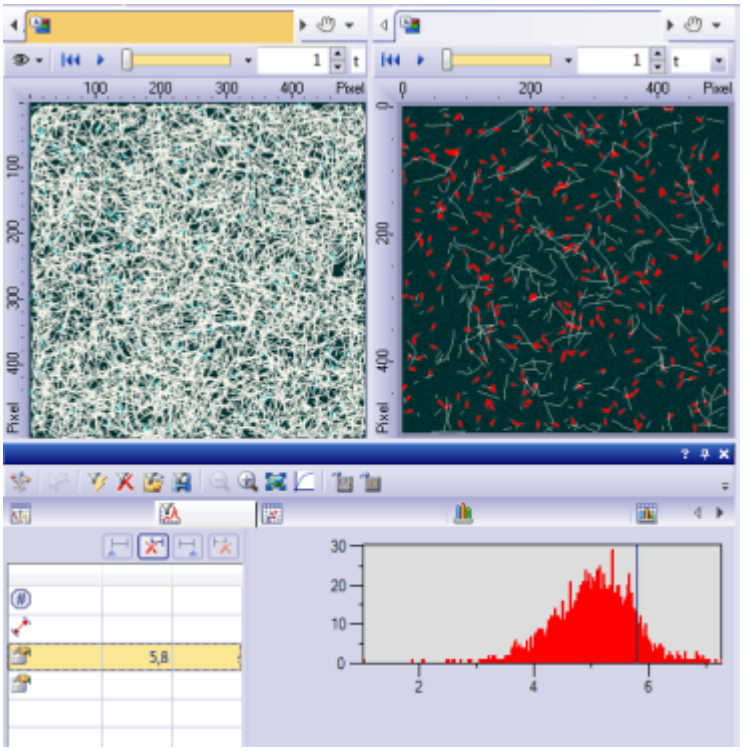


At the bottom you can see the *Track Object Measurements* results view where the velocity and the track are listed for all of the detected objects. The *Pan and Zoom to Object* button is clicked (**1**). This indicates that the corresponding selection mode is active. The object in the first row is selected both in the table (**2**) and in the image window (**3**).

12.3.8. Filtering tracks

Many tracks can be analyzed in a time stack at the same time. Define a filter to limit the number of tracks that are shown.

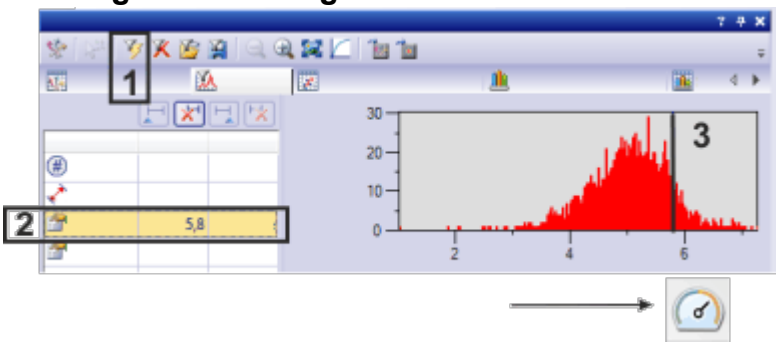
Task: You have tracked very many objects in a time stack. You are only interested in the fast objects. Hide all of the slow objects.



The image on the left has so many tracks that the results of the object tracking process can't be interpreted. A track filter that only displays the tracks for fast objects has been applied in the image on the right. The other tracks have been excluded by the filter.

1. Load or acquire a time stack that shows the moving objects that you want to track.
2. Track the objects in the time stack.
3. In the *Object Tracking Results* tool window, click the *Track Filter* tab.
   - In the table on the left-hand side of the *Track Filter* tab, you can see a list of all of the measurement parameters that are currently selected for track measurements. The corresponding filter ranges are listed next to the measurement parameters.
   - If the measurement parameter that you want to use for the track filter isn't shown in the list, activate the *Object Tracking Results > Track Measurements* results view. Click the *Select Track Measurements* button.
     If you want to filter by object velocity, for example, select the *Mean (Track Velocity)* measurement parameter.

Defining the filter range



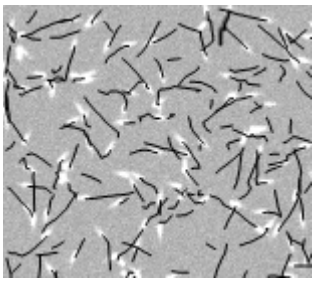
Use the *Track Filter* tab in the *Object Tracking Results* tool window to hide tracks.

4. Click the *Toggle Track Filter* button (**1**) to switch on the track filter.
   - The button will appear clicked.
   - The *Track Count* group in the *Object Tracking* tool window indicates that a track filter is active. In the *In filter ranges* field, the number of tracks that fall within the defined filter range are shown.
5. Select the *Mean (Track Velocity)* (**2**) measurement parameter in the table.
   - A histogram with the velocity of the physical tracked objects is displayed to the right of the table containing the measurement parameters. The velocity is plotted on the X-axis and the number of tracks is plotted on the Y-axis.
6. Double click in the *Min* field. Enter the lowest velocity at which you want an object still to be taken into account.
   - A line appears in the histogram (**3**). You can still drag this line to the left and right in the histogram. This immediately changes the current minimum value.
   - In the image window, the result of the filtering can be seen straight away. All of the tracks that are outside the defined filter range will be excluded.
   - In the results views in the *Object Tracking Results* tool window, all of the measurement values that belong to the hidden tracks are also hidden. You can show these measurement values again at any time without having to repeat the object tracking process.

Note: An object filter that has been defined is not automatically deactivated when you repeat the object tracking process. If no objects are shown in an image window, make sure that the track filter is deactivated.

12.3.9. Classifying tracks

Task: You have tracked objects on a time stack. They tracks have different lengths. You want to know how many short and how many long tracks there are in the time stack. Is there a correlation between the length of the tracks and the speed of the objects?



The illustration shows a frame in a time stack. The detected tracks are colored black.

The following process flow chart displays the basic steps of the process.

Defining a classification scheme

A classification scheme specifies which measurement parameters are used for a classification. It defines the number of classes and the ranges of the individual classes.

Classifying tracks

Apply the classification scheme to the tracks.

Defining, viewing and saving the results

Select the required measurement parameters for the classes. Use the *Object Tracking Results* tool window to view the results.

Preparing for the classification

1. Load or acquire a time stack that shows the objects that you want to track.
2. Track the objects in the time stack.

Defining a classification scheme

You don't have to define a new classification scheme every time you track an object. The classification scheme that was last selected is usually used automatically.

3. In the *Object Tracking* tool window, click the small arrow next to the *Start Tracking* button. Select the *Track Classification Options* command from the context menu to open the *Options > Tracking > Classification* dialog box.
4. In the *Current Classification* group, click this button.
   - The *Define 'One parameter' Classification* dialog box opens.
5. Enter a descriptive name for the new classification in the *Name* field, *Length classes* for example.
6. Select the *Track Length* entry in the *Measurement* list.
   - Only the measurement parameters that are displayed as measurement parameters for the track measurement in the *Object Tracking Results > Track Measurements* results view are shown in the list. The *Track Length* parameter

is an exception. This measurement parameter always appears in the list.

- If the measurement parameter that you want doesn't appear in the *Measurement* list, click this button to open the *Select Track Measurements* dialog box. Add the required measurement parameters to the list of measurements that are computed for all of the tracks.

7. Click the *Automatic Classification* button to switch to the *Automatic Classification* dialog box.
8. In the *Automatic Classification* dialog box, click the *Get Min./Max. from Image* button. Then the smallest and largest value of the selected parameter that has been entered in the *Minimum* and *Maximum* fields, will be used.
   - In this way, you'll be certain that all of the objects in the image can be assigned to one of the classes that have been defined.
9. Enter the value 2 in the *Number of classes* field, and in the *Scale* field, select the *Linear* entry.
10. Close the *Automatic Classification* dialog box with *OK*.
    - By doing this, you have defined two length classes.
    - You return to the *Define 'One parameter' Classification* dialog box.

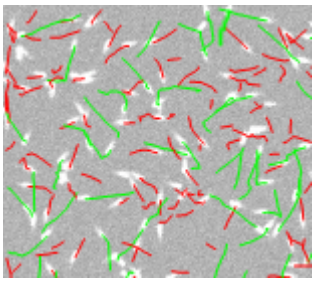
Classifying tracks

11. In the *Define 'One parameter' Classification* dialog box, click the *Classify* button.
    - All of the tracks in the active time stack are now sorted either into the short tracks or the long tracks class.
12. Close the *Define 'One parameter' Classification* dialog box.
    - In the *Options > Tracking > Classification* dialog box, the new classification is active in the list. You can now use this classification for other tracking other objects as well.
13. Close the *Options* dialog box with *OK*.

Defining, viewing and saving the results

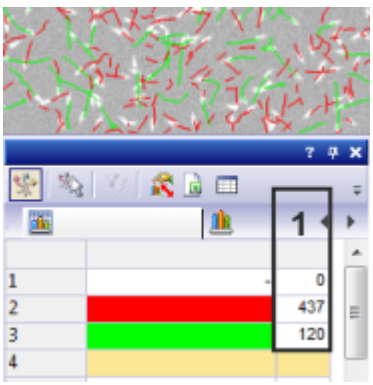
1. The tracks in the image window can be displayed in the color that corresponds to their class.

   If the color of the tracks in the image window doesn't change after the classification has been performed, check the track display settings.
   - To change the way the tracks are displayed in the image window, click the small arrow next to the *Start Tracking* button in the *Object Tracking* tool window. Select the *Track Display Options* command in the context menu to open the *Options > Tracking > Display* dialog box. Select the *Color defined by > Classification* option.



After the classification has been performed, all of the short tracks are colored red and all of the long tracks are colored green in the image window.

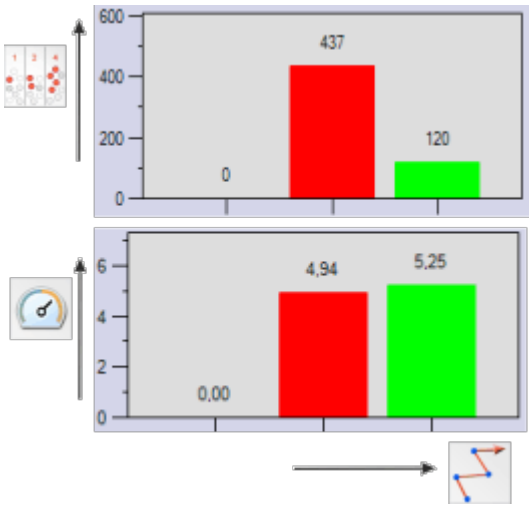
2. In the *Object Tracking Results* tool window, activate the *Track Class Measurements* results view.
3. Click the *Select Track Class Measurements* button and in the *Select Track Class Measurements* dialog box, add the *Track Count*, *Mean (Mean (Track Velocity))* and *Track Class Name* measurement parameters.
   - The *Track Count* parameter delivers the values you are looking for in the task: the number of objects found in each class.
   - With the *Track Class Name* parameter, you write the name and the color of the class in the results sheet, as well. You should, without fail, adopt this parameter in the results sheet to make it possible to assign the measurement results correctly to the individual classes. You can also show these parameters in the *Track Measurements* and *Track Object Measurements* results sheets. Then, in the results sheet, you'll be able to immediately recognize to which class each of the individual objects and tracks belongs.
   - The *Mean (Mean (Track Velocity))* parameter tells you the mean speed of the objects in a class.
4. Close the *Select Track Class Measurements* dialog box.
5. Go to the *Track Class Measurements* results view in the *Object Tracking Results* tool window to take a look at the class results in a table.
6. Then in the *Object Tracking Results* tool window, activate the *Track Class Histogram* results view to have the class results displayed as a bar chart.



In the illustration, you can see the resulting image and both of the length classes. The column (**1**) shows the number of long (green) and short (red) tracks that was being looked for.

7. Then in the *Object Tracking Results* tool window, activate the *Track Class Histogram* results view to have the class results displayed as a bar chart.

8. Select the *Track Count* entry in the *Measurement* list.
   - The histogram shows the number of tracks per class. This tells you how many short and how many long tracks there are in the active time stack.
9. Select the *Mean (Mean (Track Velocity))* entry in the *Measurement* list.
   - The histogram now shows the mean object velocity per class. The objects were sorted into classes according to the length of their tracks. This allows you to visualize a potential correlation between the velocity of the object and the length of it's track.



You can visualize different measurement parameters for the individual classes in the *Track Class Histogram* results view.
The top histogram shows the number of tracks per class. The current time stack has significantly more short than long tracks.
The bottom histogram shows the mean velocity of the moving objects by class. The speed of the two objects is roughly the same. This tells us that the different track lengths are not caused by the differing velocities of the objects.

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13. Overview - Experiment Manager

What exactly is the Experiment Manager?

You can use your software to implement complex acquisition processes. Use the Experiment Manager to define and run complex experiments involving image acquisition with your software. You can always re-use existing experiment plans or adapt them to new conditions. You can acquire multi-dimensional images during the experiment. If your microscope has motorized hardware components, you can control these with the software during the experiment. Or you can use an RTC (Real Time Controller), to use external devices in your experiments.

The idea behind the Experiment Manager

The Experiment Manager is a graphic Process Manager

You can create a graphic experiment plan with the *Experiment Manager* tool window. This experiment plan contains a series of commands, the acquisition of images for example, that are carried out one after the other.

Example: Use the Experiment Manager to acquire several multi-channel fluorescence images of a certain position on the sample at certain intervals.

How the Experiment Manager differs from the Process Manager

Just like the Experiment Manager, you can use the *Process Manager* tool window to handle complex acquisition processes. The Experiment Manager can be used as an alternative to the Process Manager. It's more intuitive for more complex processes and offers you more options.

- In the *Experiment Manager* tool window, you can trigger a particular device at a particular time using the RTC to add a chemical to your sample, or to heat or illuminate your sample, for example.
- Only the Experiment Manager allows you to define loops within loops. This makes it possible to repeat a fast time stack several times at particular intervals.
- The Experiment Manager allows you to use streaming to attain shorter intervals between two separate image acquisitions in a time stack than are possible with the Process Manager.

Prerequisites for using the Experiment Manager

Prerequisite: The *Experiment Manager* tool window is only available with the highest software package.

The system has been configured.

Make sure that your software is correctly configured.

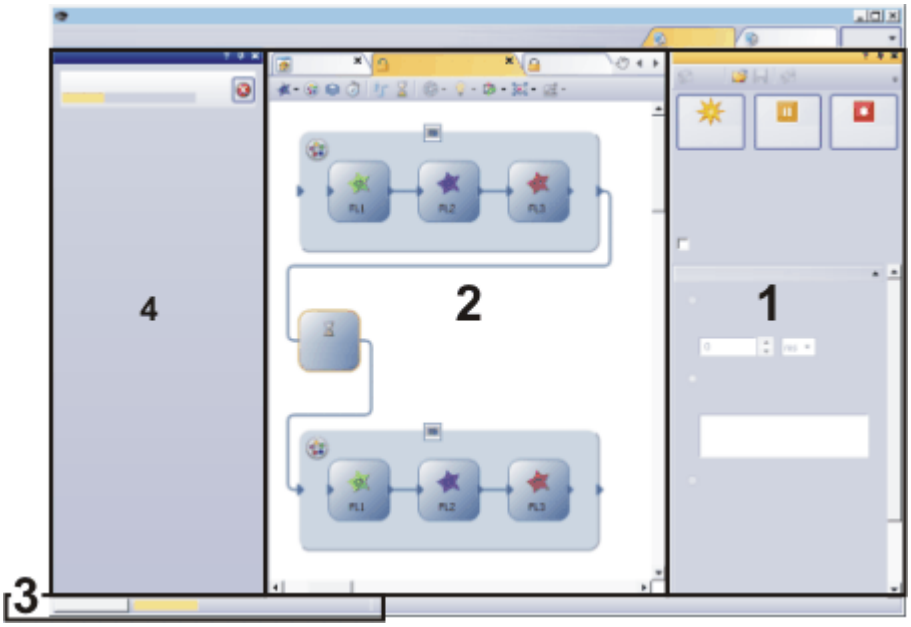
The observation methods have been defined.

When you want to acquire multi-channel fluorescence images, it makes sense to define observation methods for your color channels before you define the experiment. Only when you've defined an observation method can you assign a fluorescence color to the individual color channels when acquiring fluorescence images, for example.

You can find step-by-step instructions on the definition of observation methods for fluorescence acquisition here.

Experiment Manager's user interface

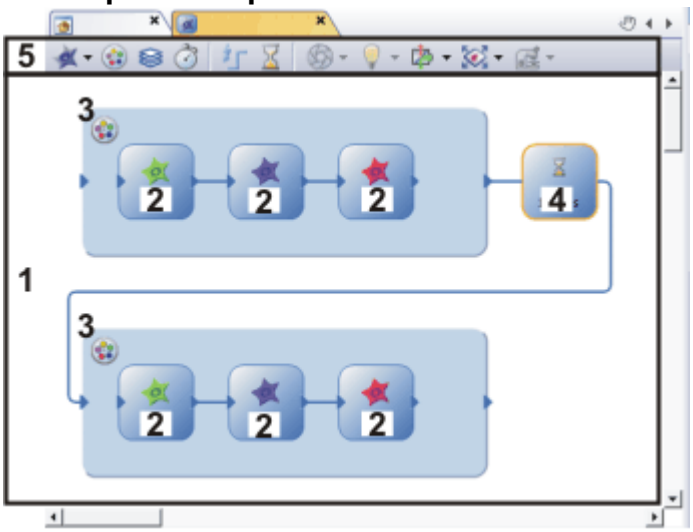
The Experiment Manager is composed of the *Experiment Manager* tool window and an experiment plan.



The Experiment Manager is composed of the *Experiment Manager* tool window (**1**) and an experiment plan (**2**). When you run an experiment, a progress bar is displayed on the left of the status bar at the bottom of the monitor (**3**). When an experiment is running over a long period of time, you can also display the *Task* tool window (**4**) to get more information about the progress of the experiment.

Use the *Experiment Manager* tool window to create an experiment plan, to edit an existing experiment plan or to start an experiment plan. To keep a good overview of the flow chart in the experiment plan, specify the settings for the commands you use in the *Experiment Manager* tool window.

The experiment plan



The illustration shows the elements on the user interface that belong to the experiment plan. The experiment plan is a document that is displayed in exactly the same way as images and other documents in your software's document group.

A document of the *Experiment plan* type is essentially a canvas on which you define the experiment by creating a flow chart (**1**).

An experiment plan contains particular commands, concerning image acquisition, as a rule. Each command is graphically displayed in the experiment plan with its own icon. The example displayed above contains the icons for a fluorescence image acquisition (**2**), the icons for the creation of a multi-channel image (**3**) and the icon for the *Wait* command (**4**).

The commands are connected with a line while an arrow clearly defines the order of the commands.

Experiment plans have their own toolbar in the document window itself (**5**). You can find all the commands that you can use in the experiment on this toolbar.

You can find the description of the *Experiment plan* toolbar here.

Activating the experiment plan in the document group

Note: When you're running an experiment, the acquired images are displayed in the document group, as a rule. In doing so, they hide the experiment plan.

If you are working in the experiment plan, click the *Keep Experiment Visible* button. You can find this button on the *Experiment Manager* tool window's toolbar. Now, the experiment plan will be shown in its own document group. The document group will automatically appear when you start the experiment. This way you make sure that the experiment plan remains visible so that you can work on it easily.

Reducing the size of the experiment plan

If your experiment plan contains a lot of commands, you can reduce its size to maintain an overview.

Use the *Zoom Out* and *Zoom In* buttons on the *Zoom* toolbar. The zoom factor of the experiment plan is displayed on the *Zoom* toolbar. The maximum zoom factor is 100%.

Alternatively, activate the experiment plan. Hold the [Ctrl] key. Now you can use the mouse wheel to zoom in and out of the experiment plan.

General process flow

You can use the Experiment Manager for two different types of tasks.

Defining and running a new experiment

The following process flow chart displays the basic steps of the process.

You can find step-by-step instructions on how to define some typical experiments here.

1. Preparing the experiment

Think through the experiment.

Make sure that all hardware components you want to use are registered in your software's device list and configured in the device settings.

Check whether the observation methods that you want to use have been defined.

Select the exposure time and optimize the image quality in the live-image.

▼

2. Defining the acquisition settings 

In the *Acquisition Settings > Saving > Process/Experiment* dialog box, specify whether and where you want the resulting images to be saved.

▼

3. Setting up the new experiment plan 

In the *Experiment Manager* tool window, click the *New* button.

▼

4. Defining the experiment plan

Add the commands for the image acquisition and the control of the hardware components to the experiment plan. Each command is represented by a graphic element that you can arrange on the canvas however you like.
Connect all commands unambiguously with each other.

▼

5. Making settings for the image acquisition and hardware control

Select each command in your experiment plan one after the other and make the necessary settings in the *Experiment Manager* tool window.

6. Running the experiment

Test your experiment while you're defining the experiment plan. To do this, click the *Start* button in the *Experiment Manager* tool window.

7. Saving the experiment plan

In case you want to run the same experiment again, with a different sample for example, save the completed experiment plan as an OEX file.

Using an existing experiment plan

You can load an existing experiment plan at any time and run the experiment again. The following process flow chart displays the basic steps of the process.

1. Defining the acquisition settings

In the *Acquisition Settings > Saving > Process/Experiment* dialog box, specify whether and where you want the resulting images to be saved.

2. Loading the experiment plan

Load a OEX file in the document group.

3. Adjusting the experiment plan

Select each command in your experiment plan one after the other and make the necessary settings in the *Experiment Manager* tool window. Change the exposure time for the acquisition of fluorescence images, for example.

4. Running the experiment

In the *Experiment Manager* tool window, click the *Start* button.

5. Saving the experiment plan

Decide whether you want to save the changed experiment plan.

Recovering data

Use the *Data Recovery* dialog box to recover images that were currently acquired and not yet saved when either the computer or your software crashed.

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13.1. Toolbar - Experiment plan

The experiment plan is a document that is displayed in exactly the same way as images and other documents in your software's document group. The experiment plan contains its own toolbar with all the commands that you can use in an experiment plan.

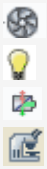
Adding commands to an experiment plan

Click a button on the experiment plan's toolbar to select the corresponding command. Now, you can insert the selected command in the experiment plan by clicking on the canvas.

Overview of the buttons

The following table lists the buttons which are available on the toolbar.

	Image Acquisition	The image acquisition is the basis for every experiment. Click this button to add an image acquisition to the experiment.
	Multichannel Group	You can combine a series of fluorescence images into a multi-channel image.
	Z-stack Loop	Add a Z-stack acquisition to your experiment.
	Stage Loop	Move the stage to different positions on the sample during the experiment. You can use the position list that you defined in the *Stage Navigator* tool window, or you can define your own positions for the experiment on the sample's overview image.
	Time Lapse Loop	Add a time stack acquisition to your experiment.
	Digital Port	Trigger a device remotely before or after the image acquisition. You can use this to add a chemical or to illuminate your sample with light of a particular wavelength or intensity, for example.
	Wait	Integrate a delay between two image acquisitions.
	You can use the following evaluation methods in your experiment, to analyze the acquired images straight away. Click the small arrow next to the button to open a menu. The button for the last used command is displayed on the toolbar.	
	Ratio	You can use the *Ratio* command to measure the change in the concentration of calcium ions in a time stack, for example.
	Intensity Profile	Use the *Intensity Profile* command. An intensity profile shows how the intensity within one, or within several image segments (ROIs), changes over a period of time or over the different Z-positions.
	Extended Focal Imaging (EFI)	Use this command to calculate an EFI image for a Z-stack after it has been acquired.
	Macro	You can use this command to process the images you have acquired using a macro. You can use this option to automate

		functions like deconvolution or a neural network analysis.
	Hardware components	Usually various different devices, such as a camera and microscope, belong to your system. These hardware components can be controlled with your software in some systems. You can use these hardware components in an experiment and open or close a shutter, for example. Prerequisite: A hardware component can only be controlled by your software during an experiment when it's been correctly registered and configured in your software.
	IX3 FRAP	When you use a FRAP system, you can bleach particular areas on your fluorescence samples with a laser.
	TIRF	When you use a TIRF system, you can define the laser positions for the TIRF illumination and thus the penetration depth in one command.
	Move XY	Use the *Move XY* command to specify a different position on the sample.
	Move Z	Use the *Move Z* command to move the stage up or down.
	Autofocus	Use the *Autofocus* command to focus the sample before the image acquisition.
	Z-Drift compensation	Use the *Z-Drift Compensation* command to compensate for unwanted movement of the Z-drive.
	ZDC Dichroic Mirror	If you are using an *IX3-ZDC2* ZDC device, you can move the dichroic mirror with the *ZDC Dichroic Mirror* command. This directs the laser beam onto or away from the sample.
	Experiment Templates	There are a number of predefined typical experiment plans that you can use as a basis for your experiment plans. You can save elements of your experiment plans as building blocks. This makes it easy to use them again.
	*Keep Experiment Visible*	Click this button to display the experiment plan in its own document group. The document group will automatically appear when you start the experiment or switch to live mode. This way you make sure that the experiment plan remains visible so that you can work on it as soon as the experiment has finished. The button is active when this mode is active. You can recognize this status by the button's colored background. Release the button if you need more space for the display of the images. The acquired images are now displayed in the same document group as the experiment and cover up the experiment plan.

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13.2. Working with the Experiment Manager

Use the Experiment Manager to define and run complex experiments involving image acquisition and image analysis with your software.

Defining a new experiment

The following instructions guide you step by step through the definition of a typical experiment. The complexity of the experiments described increases from example to example.

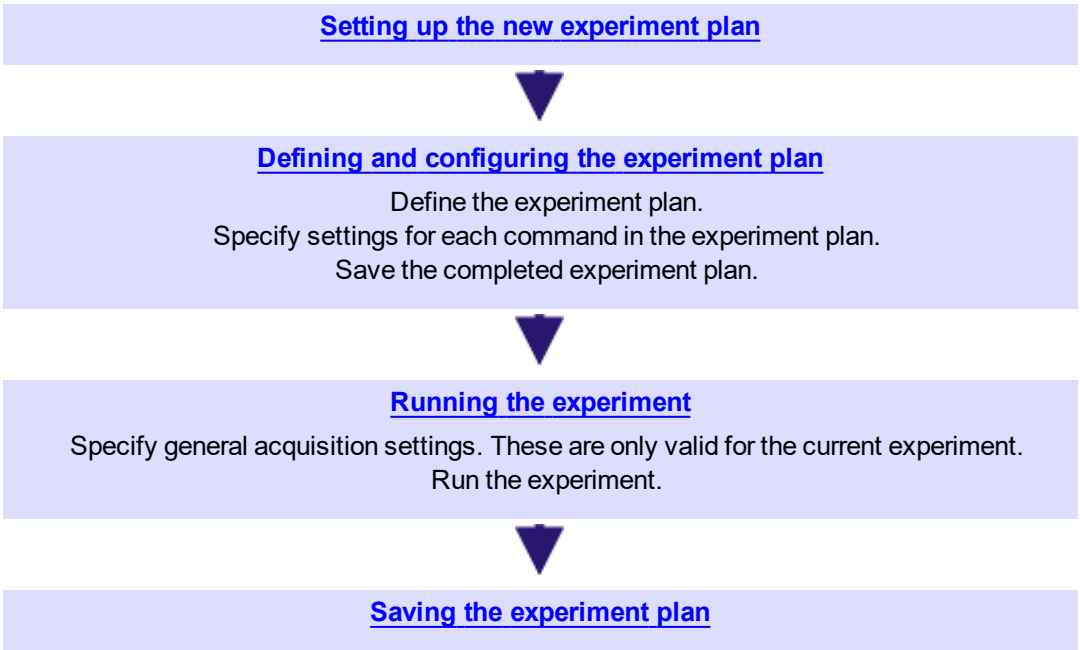
13.2.1. Acquiring fluorescence images

Example: Your sample has been stained with the DAPI, FITC, and TRITC fluorochromes. Define an experiment plan for acquiring several fluorescence images and run the experiment.

Preconditions

1. The system has been configured.
2. You have defined suitable observation methods for your color channels.
   - You can find step-by-step instructions on the definition of observation methods for fluorescence acquisition here.

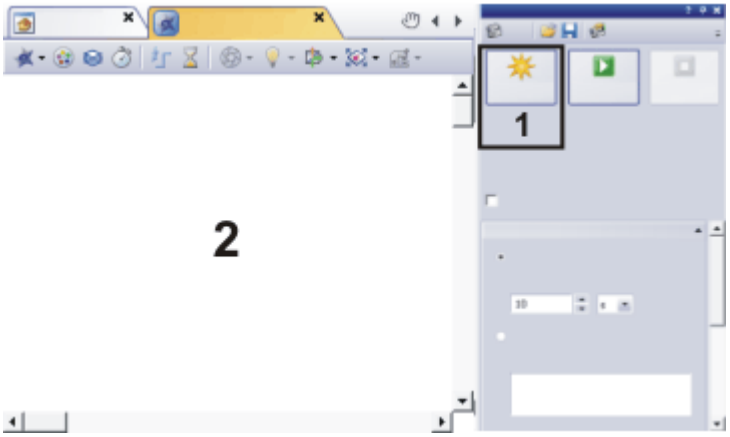
The following process flow chart displays the basic steps of the process.



Setting up the new experiment plan

1. If necessary, use the *View > Tool Windows > Experiment Manager* command to show the *Experiment Manager* tool window.

2. In the *Experiment Manager* tool window, click the *New* button to create a new experiment.
   - This automatically creates a new document of the *experiment plan* type in the document group.
   - The experiment plan contains its own toolbar with all the commands that you can use in an experiment plan. Exactly which commands appear on the toolbar depends on your system configuration.
   - In the document group, experiment plans are identified by this icon  in the header.
   - The default name for the experiment plan is *Experiment <sequential No.>*. You can change the experiment plan's name to anything you want when saving it. A small asterisk next to the name indicates that the document hasn't been saved yet.
     Please note that the name of the experiment plan isn't linked to the experiment name that you enter in the *Experiment Manager* tool window. The entry in the *Experiment name* field is, by default, incorporated into the names of the images that you acquire with the experiment plan.



Click the *New* button (**1**) to create a new empty experiment plan (**2**).

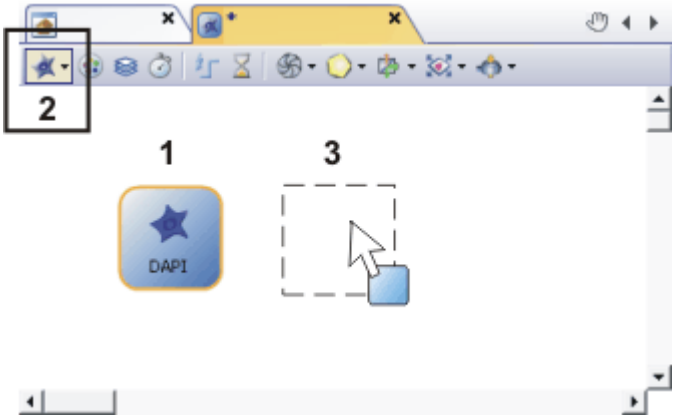
Defining the experiment plan

3. Define the first image acquisition command.
   Click the small arrow next to the *Image Acquisition* button to open a menu. Select the observation method that you want to use for the first image acquisition, *DAPI* for example.
   - All of the observation methods that are currently defined in your software are listed in the menu.

Note: When an experiment starts, the currently set objective and the camera are used during the whole experiment. If you have defined settings for the camera or the

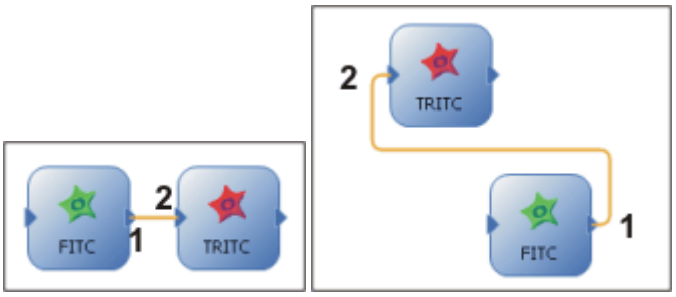
objective in an observation method and you then add this observation method to an experiment plan, these settings are not automatically adopted in the experiment. Select the camera and the objective you want before starting the experiment. To do this, select the corresponding observation method in the *Observation Methods* group in the *Microscope Control* tool window.

4. Click in the canvas on the position where you want to place the image acquisition command with the DAPI observation method in the experiment plan.



The experiment plan already contains the image acquisition command with the DAPI observation method (**1**).
Click the *Image Acquisition* button to add another image acquisition command to the experiment plan (**2**).
You can now move the image acquisition command on the experiment plan with your mouse (**3**).

5. Add the other two commands for image acquisition with the FITC and TRITC observation methods as well and arrange the commands in a row.
   - The three commands have to be connected to each other with a line. This line has an arrow which unambiguously defines the order of the commands. Depending on where you placed the commands in relation to each other, they may already be connected.
   - The experiment plan is constantly checked for errors by default. If the two image acquisition commands aren't connected, a yellow warning sign appears at the top left of the experiment plan. Move your mouse pointer over the warning sign to display a description of the syntax error that was found.
6. You can define the connector between two commands in the experiment plan manually. To do so, create a connector by dragging one of the control points on the edge of a command to a control point on the following command. When doing this, you always have to connect an output control point (on the right of the command) with an input control point (on the left of the command).
   - Your software automatically draws the connector in the experiment plan. When you move commands in the experiment plan, the connector between these commands is automatically adjusted.



Both experiment plans show the same experiment. First, an image is acquired with the FITC observation method and then an image with the TRITC observation method. The green *FITC* command's output control point (**1**) is correctly connected to the red *TRITC* command's control point (**2**).
The connector has been selected in both experiment plans and is therefore displayed in color.

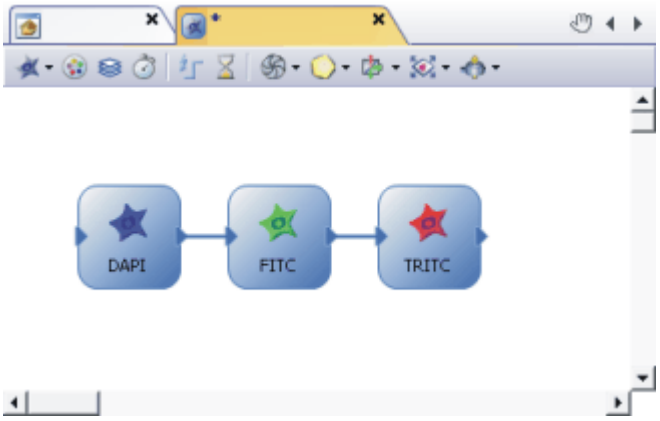
Configuring the experiment plan

Define the exposure time and other acquisition settings for the image acquisition commands.

7. Select the DAPI command in the experiment plan.
   - In the *Experiment Manager* tool window, the *Image Acquisition*, *Camera Settings*, and *Display* groups are now shown.
8. Click the *Apply Settings* button. You can find the button in the *Image Acquisition* group in the *Experiment Manager* tool window.
   - The DAPI observation method is specified on the microscope.
9. Click the *Live* button in the toolbar at the top of the *Experiment Manager* tool window to switch to live mode. In the live-image, check the exposure time and focus on the sample.
   - Note: The live-image covers the experiment plan in the document group.

     When you turn off live mode again, the live-image is closed by default and you see the experiment plan again.

     If you've selected a different setting for the live-image, you can activate the experiment plan after ending the live mode, in the *Gallery* tool window, for example.
10. Set the resolution and the bit depth in the *Experiment Manager* tool window. Set the exposure time and the sensitivity or gain for an optimal image.
    - Note: You can also use the *Camera Control* tool window to optimize the live-image. The acquisition settings in the *Camera Control* tool window are not, however, transferred automatically to the selected command in the experiment plan. To do this, click the *Get Settings* button in the *Experiment Manager* tool window.
11. In the same way, define the acquisition settings for the FITC and TRITC commands. You can make different settings for each image acquisition command. For example, expose the different fluorescence images differently to even out the differences in the light intensity.



The experiment plan contains three individual image acquisition commands. Because the image acquisition commands are linked to an observation method, before the image acquisition your system automatically takes on the settings you defined in the observation method. For the acquisition of fluorescence images, the required mirror cubes are brought into the light path, for example.
The experiment will produce three fluorescence images.

Running the experiment

Specify some general acquisition settings for the running of the experiment. These acquisition settings are not saved together with the experiment plan.

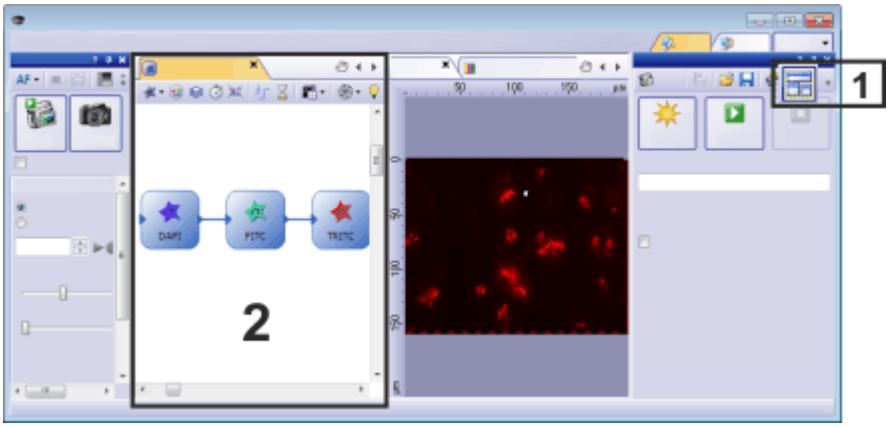
1. Click the *Acquisition Settings* button, located in the *Experiment Manager* tool window's toolbar.
2. Select the *Saving > Process/Experiment* entry in the tree view.

   Here, you specify whether and how the acquired images are to be automatically saved. You can have the acquired images saved in a database or in a directory of your choice. You can also switch off the automatic save process. In this case, the acquired images stay open in your software's document group after the experiment has finished.
3. Select the *Document Name > Process/Experiment* entry in the tree view.
   Here, you specify how the acquired images should be named.
4. Some camera settings, like Online Deblur for example, in the *Camera Control* tool window are set globally for the whole experiment. Just like the acquisition settings, they're not saved together with the experiment.

5. Click the *Keep Experiment Visible* button, located in the toolbar at the top of the *Experiment Manager* tool window.
   - The button is active when this mode is active. You can recognize this status by the button's colored background.

6. Click the *Start* button, located in the *Experiment Manager* tool window to run the experiment.
   - Note: You can also start an experiment when the experiment plan is not active in the document group. If more than one experiment plan is open, the last experiment that was active is always started.
   - The experiment starts immediately. Three individual fluorescence images will be acquired.

- When the *Keep Experiment Visible* button is active, a new document group will automatically be created in the experiment plan after the experiment is started. Now, the experiment plan remains visible while the experiment is running.



When the button (**1**) is active, a new document group (**2**) is automatically created for the experiment plan.

Saving the experiment plan

7. Activate the experiment plan in the document group.
8. Use the *File > Save As* command to save the experiment plan. Save the experiment plan, under the name *3_FL_Images*, for example.
   - An experiment plan will be saved in the OEX file format.
   - If you have the results of the experiment automatically saved, the experiment plan is also automatically saved so that the experiment will be as well documented as possible. The automatically saved experiment plan is named like the first image that has been acquired.

     You can find the saved experiment plan in the same directory as the other data is saved in. You can specify the storage location in the *Acquisition Settings* dialog box.

13.2.2. Acquiring multi-channel fluorescence images

Acquiring a fluorescence image with three color channels

Your sample has been stained with the DAPI, FITC and TRITC fluorochromes. Define an experiment plan for acquiring a multi-channel fluorescence image.
Run the experiment and acquire a multi-channel image.

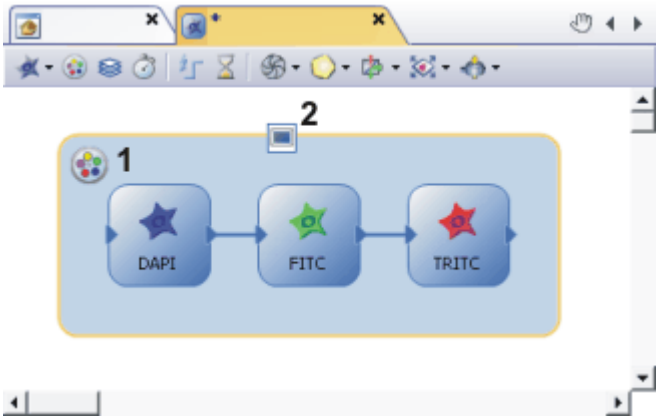
1. Load an experiment plan in which three fluorescence images are acquired one after the other.
2. Use the *File > Save As* command to save the experiment plan with a new file name.

Inserting a 'Multichannel' system template

Your software offers several predefined typical image acquisition commands. You can use these system templates to create an experiment plan quickly.

3. Click the *Experiment Templates* button. You can find the button on the experiment plan's toolbar.
   - A menu of commands that apply to experiment templates opens.
4. Select the *System Templates* command to open a list of available predefined experiment plans.
5. Select the *Multichannel* entry to insert the command for acquiring a 3 channel fluorescence image into the current experiment plan.
6. Click the canvas at the position in the experiment plan where you want to place the command.
   - Your software inserts the command for acquiring a 3 channel fluorescence image into the current experiment plan. The command is composed of four individual commands: three image acquisition commands for acquiring the individual color channels, and a command that combines the three color channels into a multi-channel image after they have been acquired.
   - Your software uses the current device configuration and the current observation methods.

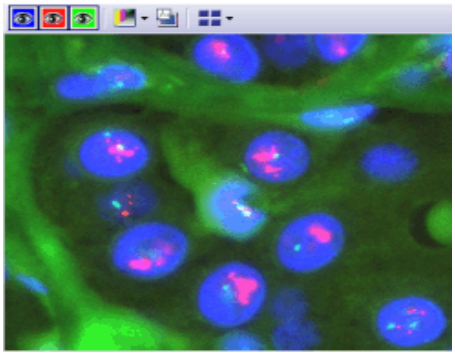
   - You usually have to adjust the predefined acquisition commands. A yellow warning sign indicates potential errors. Move your mouse pointer over the warning sign to display a description of the error that was found.
7. Select the individual commands and configure the commands in the *Experiment Manager*. You can assign a different observation method to an image acquisition command or change the exposure time, for example.



The illustration shows an experiment plan for a multi-channel image acquisition.
The name of the multi-channel group (**1**) and the status of the online display (**2**) are displayed in the experiment plan.
The experiment will produce a multi-channel image with three color channels.

8. Test the experiment. To do this, click the *Start* button in the *Experiment Manager* tool window.

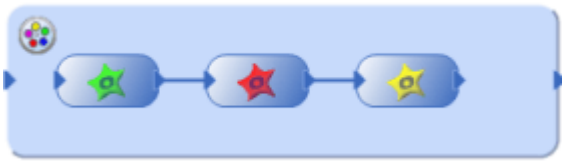
- The acquisition of the multi-channel fluorescence image starts immediately.
- The acquisition has been completed when you can again see the *Start* button in the *Experiment Manager* tool window.
- The acquired fluorescence image is displayed in the image window by default after the experiment is finished. In the image window, all three color channels are superimposed on each other so that you see all three fluorescence images at the same time.



9. In the image window, take a look at the multi-channel fluorescence image that has been acquired. If necessary, change the settings for individual commands in the experiment plan.

Defining a Z-offset for individual color channels

Usually, the focus position is different for each color channel. Extend your experiment plan and select the optimal focus position for each color channel.



You acquire a multi-channel fluorescence image with three color channels with this experiment plan.

1. Select a color channel in the experiment plan.
2. Choose the observation method that belongs to the selected color channel and switch to live mode.
3. Bring the sample into focus.
4. Select the *Use Z-Offset* check box in the *Experiment Manager* tool window. The check box is located at the top of the tool window.
   - The *Z-Offset* group at the bottom of the *Experiment Manager* tool window is now available for image acquisition commands.
5. Click the *Define Z Reference* button in the *Z-Offset* group to define the selected color channel as a reference for the Z-offset.
   - This icon is now displayed on the selected color channel in the experiment plan.

- The current Z-position is displayed next to the *Define Z Reference* button. This Z-position is used as a reference value for the Z-offset of the other color channels.
- As you can't enter a Z-offset for the reference image, the *Z-Offset in µm* field is not available as long as the reference color channel is selected.

In this experiment plan a Z-offset for the individual color channels is defined. The green color channel is used as a reference.
Select one of the other color channels to view its Z-offset values in the *Experiment Manager* tool window (**1**). In the example shown, the microscope's Z-drive is raised by 8 µm in relation to the current Z-position of the reference color channel before the acquisition of the red color channel.

6. Select the next color channel within the multi-channel group.
7. Choose the observation method that belongs to the selected color channel and focus the sample.
8. Click the *Read Z-offset* button in the *Z-Offset* group to adopt the current Z-position of your microscope stage.
   - Your software computes the difference to the reference color channel's Z-position and enters this value in the *Z-Offset in µm* field.
9. Define the Z-offset for the other color channels within the multi-channel group.
   - When you later load and run the experiment plan, focus before the acquisition of the reference color channel. The focus position of the remaining color channels is then adjusted accordingly.

Acquiring a multi-channel fluorescence image together with a transmitted light image

Expand your experiment plan and acquire a transmitted light image as well, with the brightfield observation method, for example.

1. Select the multi-channel group in the experiment plan. Use the mouse to enlarge the frame so that there's enough room inside the group for another image acquisition command.
2. Click the small arrow next to the *Image Acquisition* button to open a menu. Choose an observation method for the acquisition of a transmitted light image, e.g., phase contrast, differential interference contrast (DIC), or brightfield.

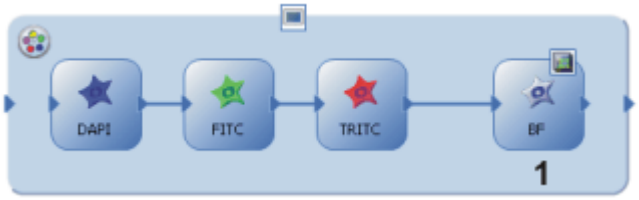
   Position the command to the right of the last fluorescence image acquisition inside the multi-channel group.
3. Connect the last fluorescence image acquisition command with the transmitted light image acquisition command. To do this, click on a control point and, while holding the left mouse button pressed, move the mouse pointer to the other

control point.

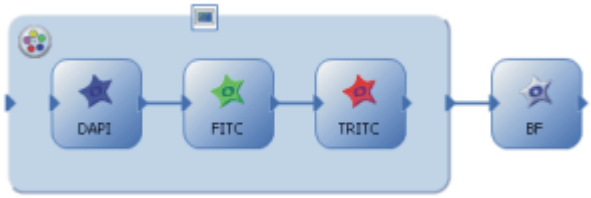
4. Select the command for the transmitted light acquisition in the experiment plan.
   - In the *Experiment Manager* tool window, the *Image Acquisition*, *Camera Settings*, and *Display* groups are now shown.
   - The *Transmission overlay* check box in the *Image Acquisition* group is available when the command selected in the experiment plan is linked to a transmitted light observation method.
5. Select the *Transmission overlay* check box in the *Experiment Manager* tool window. Now, the transmitted light image is assigned its own image layer on top of the fluorescence images.

   - This icon appears in the experiment plan on the command for the acquisition of the transmitted light image.
6. Make the rest of the settings for the acquisition of the transmitted light image.

7. Click the *Start* button, located in the *Experiment Manager* tool window to run the experiment.
   - Then, together with your fluorescence images, a transmitted light image will also be acquired and saved together with the multi-channel fluorescence image. The result of this acquisition process is a multi-layer image that you can view with the *Layers* tool window.



The illustration shows an experiment plan for a multi-channel acquisition with a transmitted light image (**1**).
The transmitted light image acquisition command is inside the multichannel group. The experiment will produce a multi-layer image with two image layers. One image layer is the multi-channel image and the second layer is the transmitted light image.



Alternatively, you can also use this experiment plan to acquire a multi-channel image with a transmitted light image. The transmitted light image acquisition command is, in this case, outside the multi-channel group. The experiment then produces two images, one multi-channel image and the transmitted light image. In this case, you can't place one images on top of the other to view the superimposition.

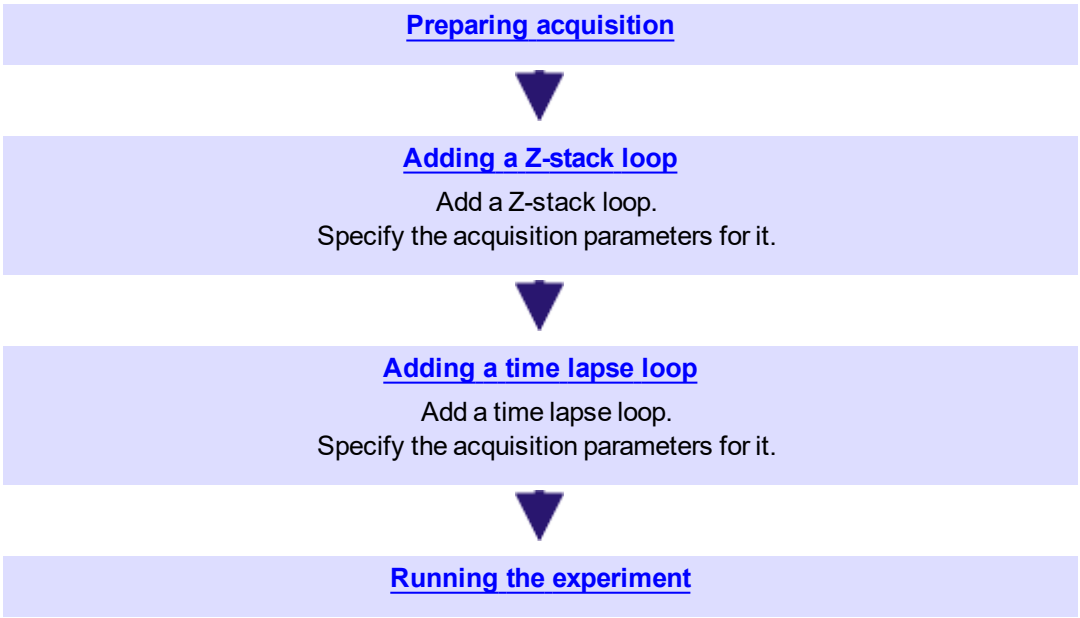
13.2.3. Acquiring multi-dimensional images

Example: Define an experiment plan for acquiring a multi-channel Z-stack. You want the acquisition of the multi-channel Z-stack image to be repeated several times at intervals of one hour.
Run the experiment.

Preconditions

- The system has been configured.
- You have defined suitable observation methods for your color channels.
- Your microscope has a motorized Z-drive. The Z-drive has been set up and calibrated.

The following process flow chart displays the basic steps of the process.



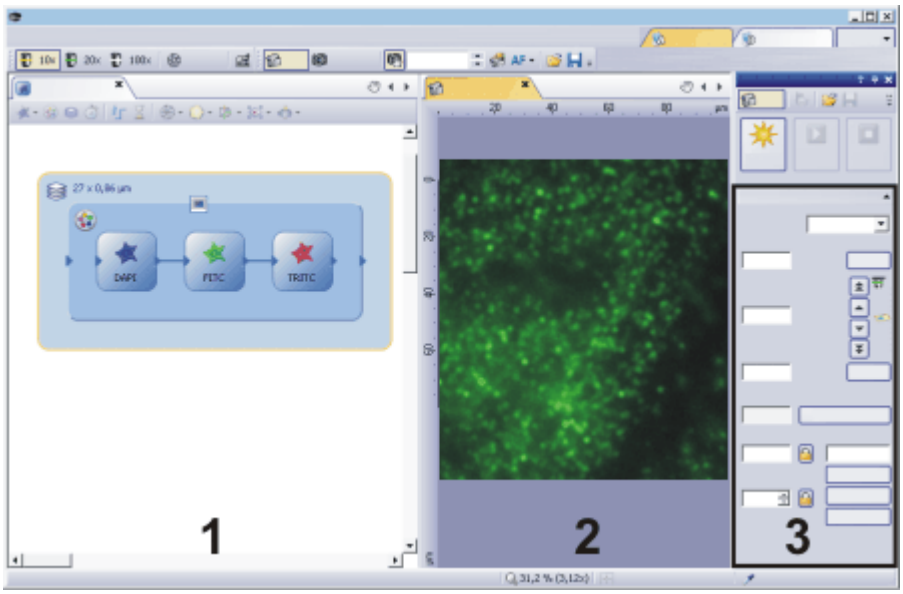
1. Load an experiment plan for acquiring a multi-channel fluorescence image. Or specify a new experiment plan.
2. Use the *File > Save As* command to save the experiment plan with a new file name.

Preparing acquisition

3. Select one of the image acquisition commands in the experiment plan, DAPI for example. Click the *Get Settings* button in the *Experiment Manager* tool window to have the DAPI observation method set on the microscope.
4. Click the *Live* button, located in the toolbar at the top of the *Experiment Manager* tool window.
5. Arrange the live window and the experiment plan in the document window so that you can see both documents at the same time. To do this, use the *Window >*

Split/Unsplit > Document Group (Right) command, for example.

6. Bring the image into focus.



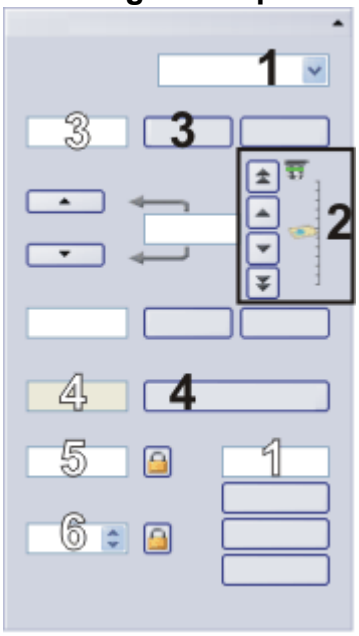
Some of the settings for the Z-stack acquisition can best be checked in the live-image. You can display the live-image (**2**) and the experiment plan (**1**) in the document window at the same time. The Z-stack loop has been selected in the experiment plan. In the *Experiment Manager* tool window, the *Z-stack* group (**3**) is now visible. Set the acquisition parameters here.

Adding a Z-stack loop

7. Click the *Z-Stack Loop* button. You can find the button on the toolbar at the top of the experiment plan.
8. Draw a frame around the multi-channel group. Your microscope's Z-drive will now be automatically moved to a different Z-position when all the commands in the Z-stack loop have been carried out.
   - The *Z-stack* group is displayed in the *Experiment Manager* tool window. Set the acquisition parameters for the Z-stack loop here.
9. Define the acquisition parameters for the Z-stack loop in the *Experiment Manager* tool window.
   - In the case being described, first the whole multi-channel image is acquired at a Z-position. Only then does the stage move to the next position.

     You can also define the experiment to acquire the whole Z-stack for one color channel first. Then the observation method is changed and then the whole Z-stack is acquired for the next color channel.

Selecting the acquisition parameters for the Z-stack loop



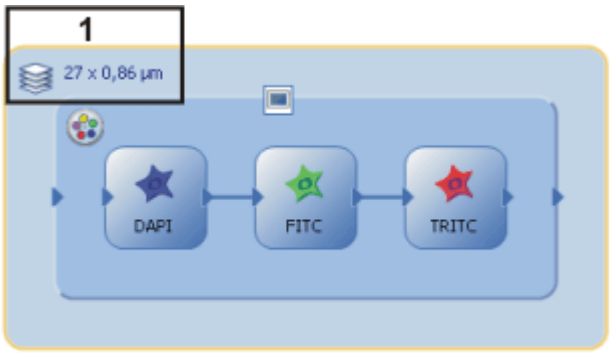
Set the acquisition parameters for the acquisition of a Z-stack in the *Experiment Manager* tool window. The acquisition parameters apply to the Z-stack loop that is selected in the current experiment plan. Use the fields and buttons with black numbers (**1**-**4**) for this. The values in the fields with white numbers are automatically calculated and updated by your software.

10. Select the *Top and bottom* entry in the *Define* list (**1**).
    - The stage's current Z-position will be shown in the *Position* (1) field. Because you've already focused, this is the focus position.
11. Using the arrow buttons (**2**), move the microscope's Z-drive up to the Z-position at which the structures that are directly under the surface of the sample are displayed in sharp focus. The buttons with a double arrow move the stage in larger steps.

    Now, move the Z-drive the same distance again in the same direction. Click the upper *Set* button (**3**).
    - The current Z-position will be adopted in the *Start* field (3).
12. Now, define the final position in exactly the same way.
    - The *Recommended Step Size* field (4) displays the distance required between two Z-positions in the Z-stack. The required distance depends on the objective's Numerical Aperture, among other things, and is automatically calculated using the Nyquist theorem. This process assures that no parts of the sample remain blurred between two frames. The higher your objective's magnification and the Numerical Aperture are, the smaller the required distance will be.
13. If necessary, release both of the buttons showing the lock icon .
14. Click the *Apply* button (**4**) located next to the *Recommended Step Size* field.
    - The *Step Size* field (5) adopts the value from the *Recommended Step Size* field.

- The *Z-Slices* field (6) now displays how many Z-positions will be moved to by the Z-stack loop. The number of Z-positions will be automatically calculated on the basis of the Start and End values, and the Z-spacing.

15. Finish the live mode.



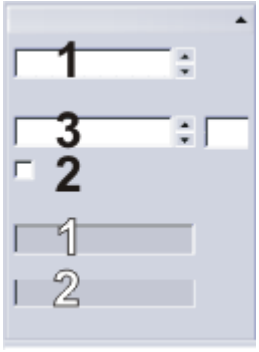
The illustration shows an experiment plan for the acquisition of a multi-channel Z-stack image. The experiment plan displays the number of Z-positions that will be moved to and the Z-spacing (**1**). In this example, 27 individual images with a Z-spacing of 0.86 µm will be acquired for each channel.

back to process flow chart

Adding a time lapse loop

16. Click the *Time Lapse Loop* button. You can find the button on the toolbar at the top of the experiment plan.
17. Draw a frame around the whole multi-channel Z-stack image. The acquisition of the multi-channel Z-stack image will now be repeated. All acquired images will be combined into a single multi-dimensional image, a multi-channel Z-stack image.
    - The *Time Lapse Loop* group is displayed in the *Experiment Manager* tool window. Set the acquisition parameters for the time lapse loop here.
18. Define the acquisition parameters for the time lapse loop in the *Experiment Manager* tool window.

Selecting the acquisition parameters for the time lapse loop



Set the acquisition parameters for the acquisition of a time lapse loop in the *Experiment Manager* tool window. The acquisition parameters apply to the time lapse loop that is selected in the current experiment plan. Use the fields with black numbers and the check box (**1**-**3**) for this. The values in the fields with white numbers are automatically calculated and updated by your software.

19. In the *Cycles* field (**1**), enter how often the command should be repeated in the time lapse loop. Enter the value 5 to acquire five multi-channel Z-stack images, for example.
20. Clear the *As fast as possible* check box (**2**).
21. In the *Interval* field (**3**), enter the time interval you want between two cycles. Enter the value 0.5 h, for example. Now, the acquisition of a new multi-channel Z-stack image will begin half an hour after the start of the previous acquisition.
    - The *Approximate minimum interval* field (1) displays the minimal time needed to carry out all the commands in the current time lapse loop. This is the amount of time that will be needed when you select the *As fast as possible* check box.
    - The *Total loop duration* field (2) displays the time needed to carry out the time lapse loop.

back to process flow chart

Running the experiment

The illustration shows a completed experiment plan for the acquisition of a multi-channel image. It consists of three nested loops. There is a multi-channel group (**1**), a Z-stack loop (**2**) and a time lapse loop (**3**).
The result is an image file.

22. Click the *Start* button, located in the *Experiment Manager* tool window to run the experiment.
    - The experiment starts immediately. Nested loops are carried out starting on the inside and working to the outside. To be exact, in this case that means:

      First a multi-channel fluorescence image is acquired.

      After this has been done, the stage's Z-position changes, and another multi-channel fluorescence image is acquired at the new Z-position.

      The Z-position keeps changing until all Z-position have been used.

      Your system waits for half an hour and then repeats the image acquisitions.
    - The acquisition has been completed when you can see the *Start* button in the *Process Manager* tool window again, and the progress bar is no longer displayed.
    - Your system's hardware components are now set as specified for the last

observation method that was used.

- The experiment produces a single multi-dimensional image.

13.2.4. Acquiring fast fluorescence time stacks

You can use the Experiment Manager to acquire very fast fluorescence time stacks. In this way, you can acquire kinetic processes with your system's highest possible time resolution.

1. Define an experiment plan with a fluorescence image and a time lapse loop.

2. Select the command for acquiring fluorescence images in the experiment plan. Select the *Streaming* check box in the *Experiment Manager* tool window.
   - Pay attention to the acquisition time that is displayed in the *Image Acquisition* group in the *Experiment Manager* tool window. The acquisition time decreases which immediately shows the effect the streaming is having.
   - Please note that streaming reduces the duration of the acquisition, the total duration can, however, can increase slightly. The total duration is displayed at the top of the *Experiment Manager* tool window in the *Experiment* group. This is because your camera requires a little time to switch the camera into streaming mode This time adds to the total duration.
   - The command for the fluorescence image acquisition in the experiment plan now looks slightly different.

On the left, you can see the command for a fluorescence image acquisition using the DAPI observation method.
On the right, the *Streaming* check box is selected. The status of the check box is indicated by a slightly changed icon in the experiment plan.

3. Select the command for the time lapse loop in the experiment plan. Select the *As fast as possible* check box in the *Experiment Manager* tool window.
4. Click the *Start* button, located in the *Experiment Manager* tool window to run the experiment.
   - All images in the time stack are now acquired at the maximum possible frame rate without waiting to be triggered by the software or the hardware. For example, you can specify an observation method that opens a shutter before the image acquisition and closes it again after the image acquisition. When you are using streaming, the shutter stays open for the whole duration of the time stack acquisition. Without streaming, the shutter would be opened before a single image acquisition and closed again.

This is how an experiment plan for the fast acquisition of a fluorescence time stack with the *TRITC* observation method looks.

13.2.5. Acquiring multi-channel fluorescence images at different positions on the sample

Example: Define an experiment plan with which you can acquire multi-channel fluorescence images at different positions on the sample. The experiment should always start at a specific stage position.
Your sample has been stained with the DAPI and FITC and fluorochromes.

Preconditions

- The system has been configured.
- You have defined suitable observation methods for your color channels.
- Your microscope has a motorized Z-drive. The Z-drive has been set up and calibrated.
- Your microscope has a motorized XY-stage. The XY-stage has been set up and calibrated.

1. Load an experiment plan that acquires a multi-channel fluorescence image or create a new experiment plan.
2. Check the settings for each image acquisition command.

Adding stage positions

3. Click the *Stage Loop* button. You can find the button on the toolbar at the top of the experiment plan.
4. Draw a frame around the multi-channel group.
   - The *Stage Loop* group is displayed in the *Experiment Manager* tool window. Use the functions in this group to define the positions on your sample where a multi-channel fluorescence image is to be acquired.
5. Move the stage to the first position and focus.
   - To move the stage, you can use the joystick or the navigation wheel in the *Microscope Control* tool window.
     If you've acquired an overview image of your sample, you can also click on the position on the sample in the overview image in the *Stage Navigator* tool window.

6. Click this button in the *Stage Loop* group to add the current stage position to the position list.
7. Select more positions for the acquisition of the multi-channel fluorescence image.

- The current number of positions is shown in the experiment plan.
- When the experiment starts, the stage will go to all positions in the position list one after the other. The experiment will be carried out at each position that is defined inside the stage loop. In this example, a multi-channel fluorescence image will be acquired at each position.

The experiment plan contains a stage loop with 8 stage positions (**1**). A 2-channel fluorescence image is acquired at each stage position.

Checking stage positions

You can go back to stage positions that have already been defined to check them and, if necessary, to delete them from the position list at any time.

8. Select the stage loop in the experiment plan.
9.  In the *Experiment Manager* tool window, click this button .
   - The *Position List* dialog box opens. It displays all currently defined stage positions.
10. Select a position in the list.
11. Click the *Go to Position* button to move the microscope stage to the selected position.
12. Click the *Delete Position* button to delete the selected position. Now, you can no longer use this position for the experiment.

Starting the experiment

13.  Click the *Start* button, located in the *Experiment Manager* tool window to run the experiment.
    - The stage moves to all stage positions one after the other. A multi-channel fluorescence image is acquired at each stage position.
    - The experiment will produce 8 multi-channel fluorescence images.

13.2.6. Adapting existing experiments

You can view and edit the settings for individual commands at any time.

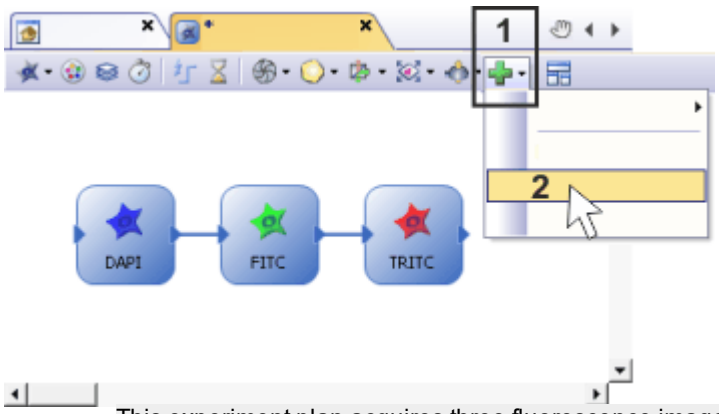
1. Should the *Experiment Manager* tool window be hidden, use the *View > Tool Windows > Experiment Manager* command to make it appear.
2. Load a saved experiment plan. Experiment plans have the OEX file name extension.

3. Select the command in the experiment plan that you want to edit, an image acquisition for example.
   - You can view and change all current settings for the selected command in the *Experiment Manager* tool window.
4. Save the experiment plan with the changed settings.

13.2.7. Creating your own experiment templates

You can save commands or groups of commands in your experiment plans as experiment templates. This makes it easy to use them again.

1. Define an experiment plan. For example, you can define an experiment plan in which three fluorescence images are acquired one after the other.



This experiment plan acquires three fluorescence images consecutively. Save the group as an experiment template.

2. Click the *Experiment Templates* button (**1**). You can find the button on the experiment plan's toolbar.
   - A menu of commands that apply to experiment templates opens.
3. Select the *Add Current Experiment to Favorite Templates* command (**2**).
   - A dialog box for saving experiment templates opens.
4. Enter a descriptive name for the experiment template in the *Name* field.
5. In the *Access* options at the bottom of the dialog box, you specify whether the experiment template is to be available only to yourself, or to all of the users.
   - The system is preset to initially make a new experiment template available only to the user who saved it. You recognize this status by the small icon ( ) next to the name.
   - Select the *Public* option. The experiment template can now be used by other users. You will be able to recognize this by the small icon next to the experiment plan's name ( ).
6. Click the *Save* button to save the new experiment template.

- You can now find the new experiment template in the *Favorite Templates* menu. Click the *Experiment Templates* button to open the menu.

- The commands in the experiment template are saved with the same settings that are currently selected for the experiment. If you change the device configuration, like deleting observation methods for example, the experiment template is not adjusted accordingly.

A yellow warning sign indicates potential errors. Move your mouse pointer over the warning sign to display a description of the error that was found.

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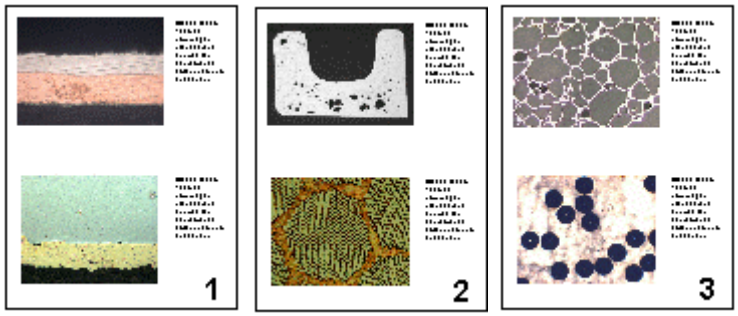
14. Overview - Report

You can create reports with your software to document the results of your work and to make them available to third parties. You can share reports as files or as printed documents.

Two programs are always involved in the creation of reports: Your image analysis software and the MS-Word application program.

You can use MS-Word 2019, 2016, 2013 (SP1) or 2010 (SP2) to create reports.



The illustration shows a report in MS-Word format.

Different ways of generating reports

The requirements for working with reports are very different depending on the user and the way you are working. There are different procedures for creating reports.

1) Creating MS-Word reports using the "Report Composer" tool window

For users who regularly create reports that are made up in the same way with a lot of images and who require these in MS-Word format.

For this, your image analysis program should be open in the foreground. In the *Report Composer* tool window, open or create a report instruction (RCI file) in which you specify which images and which page layout the report should contain. Then you create a report which is displayed in MS-Word at the touch of a button. In MS-Word you now only undertake small corrections of the report.

2) Creating and editing reports using the Olympus MS-Office Add-in

For users that want to insert images or documents that were created with the image analysis program into new or existing MS-Word documents. Also for users who want to process MS-Word reports that were created using the *Report Composer* tool window.

When you use the Olympus MS-Office add in, your image analysis program opens in the background. You use the Olympus MS-Office add-in to insert images, workbooks or charts from your software into an MS-Word document. You use what are called templates to do this. With MS-Word reports, you define **Page Templates** in the DOC or DOCX file format.

Procedure 1: Report generation using the "Report Composer" tool window

1. New report instruction

Switch to the *Reporting* layout and create or open a report instruction. Place the documents you want in it.

2. Creating a report

Create a report.

3. Working with the Olympus MS-Word add-in

In MS-Word: Have a look at the report and make small changes, if necessary.

4. Saving the report and the report instruction

If you want, print the report or create a PDF file. Save the report instruction (and the report as well, if needed).

Procedure 2: Report generation using the Olympus MS-Office add-in

1. Setting up a new DOCX file

Create a new file or open an existing document.

▼

2. Working with the Olympus MS-Office add-in

In MS-Word: Use the functions on the *Olympus* tab to add documents to the report.

▼

3. Working with the Olympus MS-Office add-in

Use the functions on the *Olympus* tab to layout the report (info stamp, detail zoom and border, for example).

▼

4. Saving the report

Save the report. If you want, print it or create a PDF file.

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14.1. Working with the report composer

The *Report Composer* tool window supports you when you are creating and updating report instructions. In this tool window, you also find the *Create* button that is used to start the report creation.

Note: Two programs are involved in the creation of reports using the *Report Composer* tool window:
Your software and the MS-Word application program. You can use MS-Word 2016, 2013 (SP1) or 2010 (SP2) for working with reports.

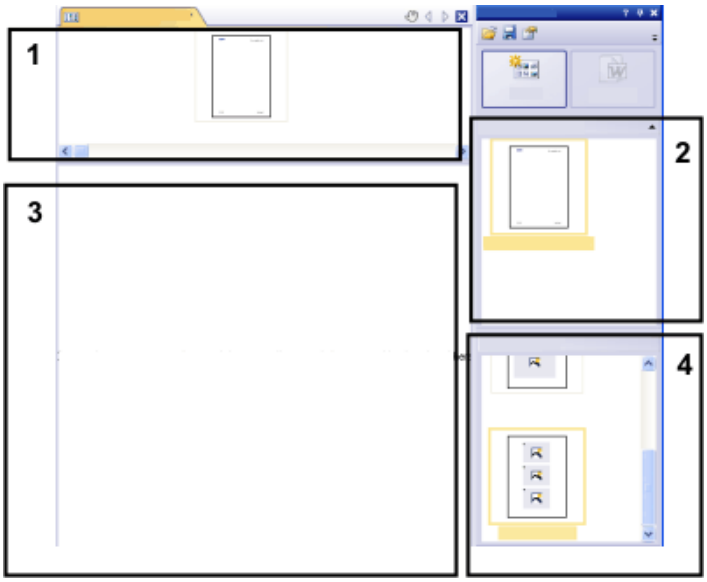
Should the *Report Composer* tool window be hidden, use the *View > Tool Windows > Report Composer* command to make it appear.

Creating a new report instruction

To create a report, first create a new report instruction in your software. You can also use a saved report instruction.

Note: The report instruction has to contain at least one registered page template.

1. Switch to the *Reporting* layout.
2.  Click the *New Report Instruction* button. You find this button in the *Report Composer* tool window.
   - A new document of the *report instruction* type will be created in the document group. This document is at the same time the workspace in which you put the report together.



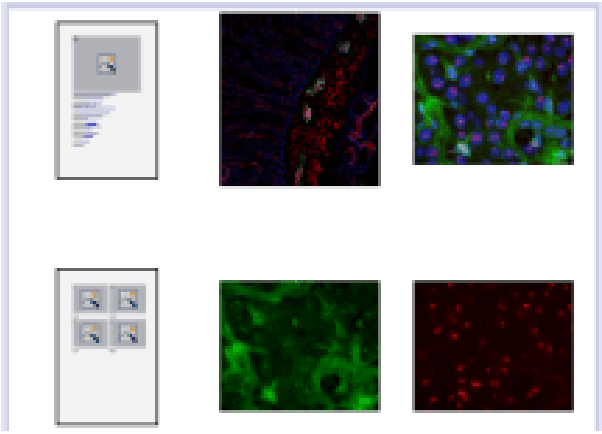
3. If no default document template has been defined: Drag the document template you want onto the upper part (**1**) of the report instruction. You find a list of the available document templates in the upper part (**2**) of the *Report Composer* tool window.
   - If a default document template has been defined, it will be automatically inserted in the upper part of the new report instruction.
   - Creating a report is also possible when you leave the upper part of the report instruction empty. In this case, the default MS-Word document template is used.
4. Drag the page templates you want onto the lower part of the report instruction (**3**). You find a list of the available page templates in the lower part (**4**) of the *Report Composer* tool window.
   - Every report has to contain at least one page template.
   - Make sure that the page templates contain the correct placeholders for the document types that you want to drag onto the report instruction. Accordingly, if your report is to contain an image and a chart, select a page template that contains one placeholder for an image and another for a chart.
   - If you want to use workbooks in your reports, MS-Excel must be installed on your PC. The minimum MS-Excel version required is MS-Excel 2010.
   - The placeholder for a workbook can also be used for an MS-Excel file. To do this, drag the MS-Excel file from MS-Windows Explorer into the report instruction. In the report instruction, MS-Excel files are shown with this icon:

     
5. Drag the documents you want onto the lower part of the report instruction (**3**).
   - In the *Reporting* layout, the *Database*, *Documents* and *Gallery* tool windows are arranged to the left of the document window. In each of the tool windows you can select one or more documents and drag them onto the report instruction. If you use the *Database* tool window, the documents don't have to

be open. It is sufficient to open the database. However, the *Gallery* tool window only allows you to select documents that are currently open in your software.

- You can also integrate MS-Word files (e.g., background information regarding the project) into your MS-Word reports. MS-Word file don't need a placeholder in the report instruction. Drag the MS-Word file from MS-Windows Explorer into the report instruction. In the report instruction, MS-Word files are shown with this icon:

- The documents must have been saved, because unsaved documents cannot be included in a report.



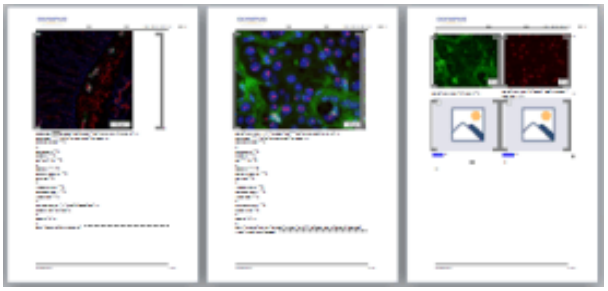
The illustration shows an example of a report instruction. In the report, two different page templates are to be used. The first page template contains a single placeholder for an image, the second page template contains two placeholders for an image. After the page template, the images that are to be inserted in the report page are displayed.

6. Check the report instruction now. You may still edit it and, e.g., delete or shift documents or select another page template.

Creating a report

1. Click the *Create* button. You find this button in the *Report Composer* tool window.
   - The report will be created. Creating a report can take some time when large reports with many images and documents are involved. Pay attention to the progress bar that is shown. The MS-Word application program will open automatically and display the new report. In the example shown below, the report has three pages. (The fact that the first page template only contains one image placeholder and two images have been added to the report instruction,

automatically leads to the creation of two report pages.)



2. If you want to, you can still make additional changes in the MS-Word application program. To do so, use the add-in from Olympus.
3. If you want to, save the report instruction and the report.

Editing a report instruction

You can make the changes described below to a report instruction. These changes do not apply to reports the have already been created on the basis of this report instruction. Therefore you must create a new report in order to see the changes you made. This will generate a new MS-Word document. Any changes that you may have made in the first version of the report is not be contained in the newly created MS-Word file.

Exchanging the document template

1. Load the report instruction that you want to edit.
   - Report instructions have the file extension RCI.
2. To delete a document template, select it and press the [Del] key on your keyboard.
3. Drag the new document template onto the upper part of the report instruction.
   - By doing so, the document template is exchanged. Please note that a report instruction can only contain one document template.
   - A report instruction must not contain a document template at all. When you leave the upper part of the report instruction empty, the MS-Word default document template will be taken.

Changing the page templates

1. Load the report instruction that you want to edit.
2. In the report instruction, select the page template you want to exchange.
3. Use the [Del] key on your keyboard to delete the selected page template from the report instruction.
   - By doing so, you only deselect the page template, no file will be deleted.
4. Drag the new page template to the position in the report instruction, where the deleted page template had been located.
   - Every report has to contain at least one page template.

Shifting the page templates

1. To shift a page template to another place in the report instruction, select it and, with the left mouse button depressed, drag it to a new position (Drag&Drop).
   - In certain cases, this may change the appearance of the report considerably. All documents that come after this page template in the report instruction will use this page template in the report.

Deleting documents

1. Load the report instruction that you want to edit.
2. In the report instruction, select the documents that you want to delete.
3. Use the [Del] key on your keyboard to delete all of the selected documents in the report instruction.
   - By doing so, you only undo the document selection, no file will be deleted.

Adding documents

You can add new documents to an existing report instruction at any time.

1. Load the report instruction that you want to edit.
2. Simply drag the new documents onto the position you want in the report instruction.
   - Dragging & dropping images onto the report instruction is possible from the *Database*, *Documents* and *Gallery* tool windows.
   - Please note the page templates must be placed before the images.

Moving documents

You can change the order in which the selected documents are arranged in the report instruction at any time.

1. Load the report instruction that you want to edit.
2. Select an image, and with the left mouse button depressed, drag it to another position (Drag&Drop).

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14.2. Working with the Olympus MS-Office add-in

When your software is installed, an add-in from Olympus is added to the MS-Word application program. When you start MS-Word, you can recognize this because the *Olympus* tab is displayed.

Note: The language on the *Olympus* tab corresponds to the language set in your image analysis software. This language can differ from the language in which the MS-Word application program is shown.

The add-ins' functions

This add-in assists you with very different tasks:

1. Inserting a document that is currently open in your image analysis software, into a MS-Word document.
2. Inserting a document that is saved locally, or is in your image analysis software's database, into a MS-Word document.
3. Inserting a field that contains information that is saved in your image analysis software into your MS-Word document. This makes sense, for example, when you want to see the acquisition date of a certain image.
4. You add one or more detail zooms to an image.
5. You change the image properties and set, for example, whether or not the info stamp and the scale bar should be shown.
6. You change the resolution of one or all images of the report. If you want to share the report, it may be sensible to reduce the resolution, thereby also reducing the file size.
7. You update all placeholders in your report. This makes sense, for example, when you've made changes to the documents in your image analysis software that the report doesn't contain yet.
8. You insert an MS-Word document into the database of your software. This command is only available if your software supports the database functionality.
9. Defining templates that you want to use for your work with reports. With MS-Word reports, you define page templates in the DOC or DOCX file format.

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Creating and editing a new template

Creating templates for MS-Word

During the installation of your image analysis software, some predefined templates were installed too. In addition to this, you can define your own templates too. With MS-Word reports, you define **Page Templates** in the DOC or DOCX file format.

The contents of a template

In a template, placeholders are set up for the documents that the report is to contain. There are placeholders for images, charts, workbooks and fields. When, for instance, the report is to contain pages that have an image at the top, and below it, a chart, you

should then set up a template, which has a placeholder for an image and a placeholder for a chart.

Note: For technical reasons, a template must consist of precisely one page. For this reason, create several separate files if you require several self-defined template pages.

14.2.1. Creating a template and adding a placeholder for a document

1. In the MS-Word application program, select the *File* tab and select the *New* entry.
2. Select the *Blank Document* option, if you don't want to use an existing page template as template, but instead want to start from scratch.
3. Decide whether you want to insert a placeholder for an image, a chart, or a workbook. On the *Olympus* tab, click one of these buttons: *Insert Image Placeholder*, *Insert Chart Placeholder*, *Insert Workbook Placeholder*. These buttons are part of the *Templates* group.
   - The placeholder you've selected will be inserted.
4. If necessary, you can change the size of the placeholder. To do so, move your mouse over a handle, then with the left mouse button depressed, drag it in the required direction. The length/width ratio remains unchanged, so that the objects won't be distorted by this action.
5. Double click a placeholder for an image, to change the default settings for its appearance.
6. If required, insert additional placeholders for images, charts or workbooks. Make sure that your template isn't longer than a page.
7. If you want to, you can insert a placeholder for a field. Additional information about a placeholder can be shown in this field, for example, the name, or the date it was set up. You will find additional information on inserting placeholders for fields further down.
8. Save your template. For page templates, use the DOC or DOCX file format. As a storage location, select the same directory that is set for your user templates or workgroup template in the software.
9. Close the file.

14.2.2. Adjusting the insertion order

The placeholders are numbered in the order in which they were inserted. Should you have initially set up placeholders for two images, have then decided to put a placeholder for a chart right at the top of the page, the insertion order would be that shown in the example on the left.

1. In this case, click the *Adjust Insertion Order* button on the *Olympus* tab, to have the insertion order numbered serially from top to bottom (see example).

14.2.3. Inserting a placeholder for a field

1. In the template, select the placeholder into which you want to insert a field.
2. On the *Olympus* tab, click the *Insert Field Placeholder* button. You can find this button in the *Templates* group.
   - In the *Placeholder* list, the name of the placeholder into which you want to insert a field appears.
3. In the *Available fields* list, select the field that is to be inserted. The entries in this list are arranged hierarchically. Click the plus sign to expand the list.
   - Two types of field are available.

     The *Document Properties* list contains fields that are, by default, in your software, managed for this document type.

     The *Database fields* list contains all of the fields that are available in the database for the selected placeholder. For this purpose, a database must have been opened.
4. Keep the *Insert Field* dialog box open. Position the mouse pointer on the location in the report where you want to insert the field.
5. In the *Insert Field* dialog box, click the *Insert* button.
   - The placeholder for a field will then be displayed. You can recognize it by the curly bracket, and by the field name shown.
6. If necessary, add placeholders for further fields. To do this, repeat the last 3 steps.
7. Close the *Insert Field* dialog box.
8. Save the template.

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14.3. Editing a report

There are several ways in which you can edit reports and optimize them for their intended use. To do so, use the add-in from Olympus.

Considerations for users who created the MS-Word report using the "Report Composer" tool window

If you want to make some changes to a report you created using the *Report Composer* tool window, before doing so, you should decide whether it will be better to make the

changes in the report (i.e., in MS-Word) or in the report instruction (i.e., in your software).
Often, it is advisable to change the report instruction first and then create a new report. Changes you make in the report instruction are valid for every subsequent report that you create with this report instruction. There are numerous changes that you can anyway, only make in the report instruction, for example, the selection of other page templates. However, changes that you make in a report are only valid for that particular report.

Changing the image properties

When images are transferred to a report, the image link is transferred as well. This makes it possible to change the image display in a report (for example, to scroll the image segment).

1. Double click the image to open the *Image Properties* dialog box. If the image is in a grouped object, first select the group and then double click the image.
2. In the *Display* group, select the *Scale bar if calibrated*, *Info Stamp* and *Border* check boxes, if these elements are to be displayed.
   - The properties of these elements can be defined in the *Options > Image Information* dialog box. Click the *Options* button to open this dialog box.
3. In the *Size* group, select one of the options that specify how large the image is to be displayed in the report.
4. If your settings should apply for all future images, click the *Set as Default* button.
5. Click the *OK* button.
   - The *Image Properties* dialog box closes. The changed image properties will be shown in the report now.

Adjusting documents

In the report, you can select a document of the "image" or "chart" type and select the *Adjust Document* button on the *Olympus* tab. You will then change over to the image analysis software, where you can edit the document and then automatically change back to the report.

Example: In the MS-Word application program, you edit a report that contains a lot of images. With a certain image you notice that an important measurement is missing. Using the *Adjust Document* button, you change over to the image analysis software, add the missing measurement and then change back to MS-Word in order to continue editing the report.

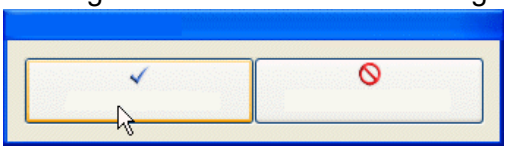
14.3.1. Adjusting an image

1. Open the report and select the image that you want to adjust. If the image is in a grouped object, first select the group and then select the image.
2. On the *Olympus* tab, click the *Adjust Document* button.
   - You switch to the image analysis software. If it was closed, it will be started and displayed in the foreground.

- The image that you want to adjust is also opened. In case it is from a database that is currently closed, the database will be opened in the background.

Note: The image analysis software is now in a special "adjust-document" mode. In this mode, you can only make certain adjustments to the image. This is why a lot of other functions are hidden.

3. Make the required change.
4. If the image information was changed: Save the image in the image analysis software.
   - Some changes made to an image don't have to be saved, e.g., when you select another frame in a multi-dimensional image. Other changes have to be saved, e.g., adding a measurement. The fact that a change has to be saved will be indicated by an asterisk shown behind the file name in the document group.
5. Click the *Update Report* button. You find this button in the *Adjust Document* message box that is shown in the foreground.



   - The MS-Word application program will now be shown in the foreground again. The edited image will be displayed. You can now continue to edit the report.
   - If your image analysis software was closed before you clicked the *Adjust Document* button, it is closed again. If any images or databases had to be opened for this command, they will be closed as well.

14.3.2. Editing a workbook in the report

Your software supports the handling of workbooks. A workbook is created, for example, when you open the *Measurement and ROI* tool window and export a results sheet.

Note: If you want to use workbooks in your reports, MS-Excel must be installed on your PC. You require MS-Excel 2010, 2013 or 2016.

Apart from the "image" and "chart" document type, reports can also contain workbooks. A workbook is imported as an Excel object in MS-Word. You can further edit it in the report.

1. In the report, double click on the workbook. If the workbook is in a grouped object, first select the group and then select the workbook.
   - You will change into the edit mode. You can recognize it by the fact that now the column headers and the row numbers are shown. In edit mode, as well as that, you can see all of the workbook's worksheets.
2. If need be, select the worksheet that you want to edit.
3. Double click the workbook in order to switch to edit mode. Make the required change.
   - When you want to format individual cells differently, select the cell and use the *Format Cells* command in the context menu.

- When you want to format the complete worksheet differently, (e.g., other font or other background color), select the complete worksheet (e.g., with the keyboard shortcut [Ctrl + A]), then select the *Format Cells* command in the context menu.
- When you want to hide a column, click on the column's header, then select the *Hide* command in the context menu.

4. Exit edit mode by clicking on any point in the report, outside the workbook.

Changing image resolution

By default, all images in a report are transferred to reports with a resolution of 192 dpi. In certain cases, it can make sense to change the resolution of individual or all images in a report For example, if you want to print the report, you can raise the resolution. Alternatively, if you want to publish the report on the Internet, you can reduce the resolution.

1. Open the report in MS-Word. Decide whether you want to change the resolution of all images or just certain images
2. If you only want to change the resolution of one individual image, select that image. If you want to change the resolution of all images, you don't have to select any.
3. On the *Olympus* tab, click the *Change Image Resolution* button.
   - The *Change Image Resolution* dialog box opens.
4. Select the option you want in the *Apply to* group. You can choose between *Selected images* and *All images in report*.
   - The *Selected images* option is inactive if no images were selected when the button was clicked.
5. Specify in the *Image Resolution* group how you want to change the image resolution. If you choose the *User-defined* option, you can enter any resolution of your choice between 96 and 600 dpi into the *DPI* field.
6. Click the *OK* button to change the image resolution.
7. Check whether you are satisfied with the changed image resolution. If not, change the image resolution anew.
   - You can first reduce the image resolution, then save the report and then increase the image resolution again. This is possible because each time that you click the *Change Image Resolution* button, the image is transferred from your software to MS-Word again.
8. Once you are satisfied with the changes to the image resolution, save the report. Take a look at the new file size in the Windows Explorer.

Updating placeholders

The *Update Placeholder* button makes it easy to have any changes made to the images after the report has been created also shown in the report. Please note that all the changes made in your software have to be saved if they are to be displayed when the *Update Placeholder* button is clicked.

Example: In MS-Word, you open a report that you created some time ago. In the meantime, you had changed a lot of images in your image analysis software (e.g., added measurements). Now, the report is to be updated so that it shows the newest version of all of the images.

1. If you only want to update one placeholder, select just that one.
2. On the *Olympus* tab, click the *Update Placeholder* button.
   - The *Update Placeholders* dialog box opens.
3. In the *Update Placeholders* dialog box, specify whether or not all placeholders should be updated.
4. Select the *Update fields linked with placeholder(s)* check box if your report contains fields which should also be updated.
5. Click the *OK* button.
   - The placeholders will be updated.

Inserting a document

You can insert a document at any position in a report. If you have, for example, created a report using the *Report Composer* tool window, and while you are viewing it, notice that you've forgotten an image, you can retroactively insert it into the report.

1. Position the mouse pointer on the location in the report where you want to insert a document.
2. On the *Olympus* tab, click the *Insert Document* button.
   - The *Insert Document* dialog box opens.
3. In the area on the left, select the source the document comes from. You have the following possibilities:
   - Select the *Open Documents* entry if you want to insert a document that is currently opened in your software.
   - Select the *Database* entry if you want to insert a document that is part of the currently selected database folder. For this purpose, the database must be opened in your software. Should you work with a version of the software that doesn't support databases, the *Database* entry is hidden.
   - Select the *File Explorer* entry if you want to insert a document that is stored on your PC or in your network.
4. Select the required document in the document preview. Click the *Insert* button.
   - The required document will be inserted into the report.
   - The *Insert Document* dialog box remains open.
5. Insert further documents now or close the dialog box.
   - The path of all documents that you inserted will be saved. That enables you to later update the inserted documents by using the *Update Placeholder* button (in case the documents were changed after they have been inserted into the report).

Inserting a field

You can insert a field into a report that describes the image in more detail. All of the values that have been saved in your image analysis software for this image can be displayed in this field.

1. Select the image in the report to which you want to insert a field. If the image is in a grouped object, first select the group and then select the image.
2. On the *Olympus* tab, click the *Insert Field* button.
   - In the *Placeholder* list, the name of the image into which you want to insert a field appears.
3. In the *Available fields* list, select the field that is to be inserted. The entries in this list are arranged hierarchically. Click the plus sign to expand the list.
   - Two types of field are available.

     The *Document Properties* list contains fields that are, by default, in your software, managed for this document type.

     The *Database fields* list contains all of the fields that are available in the database for the selected placeholder. For this purpose, a database must have been opened.
4. Keep the *Insert Field* dialog box open. Position the mouse pointer on the location in the report where you want to insert the field.
5. In the *Insert Field* dialog box, click the *Insert* button.
   - The field contents will be displayed in the report.
6. If necessary, add further fields. To do this, repeat the last 3 steps.
7. Close the *Insert Field* dialog box.
8. Save the report.

Note: Should you want to have the contents of a specific field regularly shown in your reports, you can already insert this field, (that is to say a placeholder for this field) into the page template. Then this field will be automatically filled out in every report. You can find more information on setting up a template here.

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